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Network mechanisms underlying susceptibility to helplessness and response to the antidepressant fluoxetine

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Network mechanisms underlying susceptibility to helplessness and response to the antidepressant fluoxetine

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Dedication

With love to Felix Roman and Emma Tolentino

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Network mechanisms underlying susceptibility to helplessness and response to the antidepressant fluoxetine

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Depression and post-traumatic stress disorder are common psychiatric comorbidities related to stress. These conditions are frequently treated with antidepressants such as selective serotonin reuptake inhibitors (SSRI's). However, there are individual differences in susceptibility to stress-induced psychopathologies and response to antidepressants. Therefore, there is a need to identify biologic factors that predict vulnerability to stress and response to treatment. Furthermore, few studies have examined the neural correlates of antidepressant treatment response in a stress-susceptible animal model. This dissertation had three specific aims: 1) to characterize behavioral predictors of stress vulnerability by studying three dimensions of temperament (reward dependence, novelty-specific activity and harm avoidance) before stress exposure using a stress-susceptible rat strain, 2) to identify the neural network effects of response and non-response to SSRI treatment using a stress-susceptible animal model, and 3) to determine the neurophysiologic correlates of helplessness susceptibility. This was examined via measurement of regional brain metabolic capacity and functional connectivity within relevant neural circuits, and measurements of

corticosterone and heart rate. These effects were studied in rats that underwent inescapable shock exposure followed by escape testing. Holtzman rats showed greater predisposition to helpless behavior following inescapable shock compared to Sprague Dawley and Long-Evans strains. Also, increased activity in a novel environment and low heart rate appeared to be markers of helplessness susceptibility in Holtzman rats. Limbic-cortical network effects were identified that distinguished between responders and non-responders to antidepressant treatment in the Holtzman strain. Finally, hypermetabolism of the lateral habenula and a less interactive prefrontal-limbic cortex were identified in subjects with higher susceptibility towards helplessness within the Holtzman strain. Similar findings have been reported with other depression animal models and human neuroimaging studies. These findings support that the helpless dimension of mood disorders can be accurately modeled with the Holtzman rat strain and confirm that the lateral habenula and prefrontal cortex are key regions mediating the helpless phenotype and response to SSRI treatment.

Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

1.1 LEARNED HELPLESSNESS IS A MODEL OF STRESS-RELATED PSYCHOPATHOLOGY

Learned helplessness (LH) in rodents has been widely used as an animal model of depression and post-traumatic stress disorder (PTSD). This phenomenon was first described by Overmier and Seligman in 1967. Essentially, exposure of dogs to inescapable shock reliably interfered with a subsequent escape response in a separate context. At first, dogs responded by running around in a distressed manner but eventually adopted a passive response and would lie in a corner and whine. Further studies employed a triadic design consisting of escapable stress, yoked-inescapable stress and no stress. The results indicated that the escape deficits following stress exposure were not caused by the stress itself, but rather uncontrollability was deemed to be a critical factor. In these studies, animals that did not have control over the offset of the shock developed the helpless phenotype whereas animals that could control the offset and received the same amount of shock did not develop learned helplessness (Maier *et al.*, 1982; Maier *et al.*, 1987; Seligman, 1975b). The phenomenon was termed learned helplessness because the subjects appeared to have learned that their responses were useless and that the consequences were inevitable. This parallels what often occurs in human depression when an individual loses motivation and believes that nothing he can do will alleviate his condition (Seligman, 1975a).

Learned helplessness seems to model certain aspects of human depression including attention deficits, decreased locomotion, poor performance in appetitive tasks, decreased aggression, and loss of appetite and weight (Lee & Maier, 1988; Minor *et al.*, 1984; Seligman, 1975a). In addition, LH also parallels symptoms of PTSD such as generalized fear and arousal, discrete fear of a conditioned stimulus, analgesia or

numbing and harm avoidance (Foa *et al.*, 1992). The fact that learned helplessness is a model for depression and PTSD is supported by the presence of symptom overlap between both conditions such as reward insensitivity, attention deficits and sleep disruptions (Newport & Nemeroff, 2000; Foa *et al.*, 1992; Henn & Vollmayr, 2005) (Table 1.1). Furthermore, 48% of patients with PTSD also receive a diagnosis of major depression (Kessler *et al.*, 1995).

However, the long-lasting effects of learned helplessness depend on the context in which the uncontrollable exposure and the escape testing occur. For example, if rats are restrained and passively given inescapable tail-shock, followed by escape testing in a shuttle box that delivers footshock, the LH effect appears to dissipate after two to three days (Maier, 2001; Maier & Watkins, 2005). But, if the inescapable and escapable shock share a similar context (e.g. free range of movement and footshock in both cases), the effect has been shown to last at least 28 days (Hunziker & Dos Santos, 2007). Hunziker and dos Santos argued that what an animal learns when restricted is different from what it learns when allowed to move freely and none of its responses are systematically followed by shock termination. The effects of this later paradigm more closely resemble the observations in humans since a diagnosis of depression requires persistence of symptoms for least two weeks and diagnosis of PTSD requires the presence of symptoms for one month (American Psychiatric Association, 2000b).

Table 1.1 Summary of features common to learned helplessness, depression and post-traumatic stress disorder (PTSD)

Learned Helplessness	**Depression**	**PTSD**
Passivity	Passivity	Harm avoidance
Heightened generalized fear and arousal	Introjected hostility	Heightened generalized fear and arousal
Weight/appetite loss	Weight/appetite loss	Appetite suppression
Social and sexual deficits	Social and sexual deficits	Social and sexual deficits
Reduced reward sensitivity	Anhedonia	"Emotional numbing"
Alterations in REM sleep	Alterations in REM sleep	Sleep disruption
Attention deficits	Attention deficits, impaired concentration, negative thoughts	Attention deficits, impaired concentration, intrusive thoughts

(Foa *et al.*, 1992; Henn & Vollmayr, 2005; Newport & Nemeroff, 2000; Seligman, 1975a; Shumake *et al.*, 2005)

1.2 INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO LEARNED HELPLESSNESS

Most individuals do not develop psychopathology in response to psychological stress or trauma suggesting individual differences in the response to stress (Breslau, 2001; Kendler *et al.*, 1999; Kessler *et al.*, 1995; Nestler *et al.*, 2002). For example, the lifetime prevalence of exposure to traumatic events is thought to be between 40% and 90% in the general population (Broekman *et al.*, 2007). However, the overall lifetime prevalence of PTSD is estimated at 7–12% (Broekman *et al.*, 2007) and only 6-15% of women develop depression after exposure to a stressful event (Kendler *et al.*, 1995).

Furthermore, 40-50% of the risk for depression is genetic making it as heritable as diabetes, hypertension and cancer (Nestler *et al.*, 2002); and 30% of the risk for PTSD is based on genetic factors according to a study of Vietnam era veteran monozygotic and dizygotic twins (True *et al.*, 1993). Therefore, there is a need to identify biological factors which confer the genetic predisposition to stress-induced psychopathology. Identifying these factors in humans is difficult because most human studies have examined individuals only after stress has occurred. Animal models provide a convenient way to investigate the predisposing factors underlying susceptibility to helplessness. One successful strategy has been to study rat lines selectively bred for behaviors which model human psychiatric disorders (Henn & Edwards, 1994; Shumake *et al.*, 2005). For example, congenitally helpless rats were selectively bred to display LH and showed neurological and behavioral signs similar to those seen in humans with depression and PTSD (Shumake *et al.*, 2000; Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). However, selectively-bred colonies can take years to develop and can be burdensome to maintain. Therefore, it would be beneficial if there were a commercially-available strain with increased susceptibility to LH. While there are a few reports of increased anxious or depressed behavior in some inbred rat strains such as the Wistar-

Kyoto (Braw *et al.*, 2006), to our knowledge only one study has assessed different susceptibilities to LH among different outbred strains. Wieland et al. (1986) found that Holtzman rats were twice as likely to develop learned helplessness as Sprague Dawley rats. Furthermore, Sprague Dawley and Holtzman rats showed the opposite behavioral phenotype as adults when subjected to the stress of maternal separation: hypoactive, depressive-like behavior among Holtzman (Spivey *et al.*, 2008) and hyperactive, ADHD-like behavior among Sprague Dawley rats (Colorado *et al.*, 2006).

1.3 BEHAVIORAL PREDICTORS OF HELPLESSNESS VULNERABILITY: NOVELTY SEEKING, REWARD DEPENDENCE AND HARM AVOIDANCE

Current evidence supports the relationship between individual personality traits and the predisposition to neuropsychiatric disorders such as depression and post-traumatic stress disorder (PTSD) (Cloninger *et al.*, 2006; Enns & Cox, 1997; Oquendo *et al.*, 2004; Richman & Frueh, 1997). For example, increased novelty-seeking, high harm avoidance and low reward dependence were predictive of PTSD symptom severity among Vietnam combat veterans (Richman & Frueh, 1997). Novelty seeking is viewed as a heritable bias in the activation or initiation of behaviors such as frequent exploratory activity in response to novelty (Cloninger, 1987). Harm avoidance refers to inhibition or cessation of behaviors, and manifests as anticipatory worry, fear of uncertainty, shyness with strangers, and rapid fatigability; and reward dependence is defined as responding intensely to rewarding or appetitive stimuli (Cloninger, 1987).

Animals have been selectively bred and used as disease models to characterize behavioral correlates of stress-related neuropsychiatric disorders (Pucilowski *et al.*, 1993; Vollmayr *et al.*, 2004). As mentioned previously, one such model is the congenitally helpless rat, which was selectively bred for a genetic predisposition to helpless behavior as defined by the learned helplessness paradigm (failure to escape

shock after a previous exposure to inescapable shock) (Henn & Edwards, 1994). Behavioral characteristics of congenitally helpless rats include increased novelty seeking, reduced reward sensitivity, and an impaired ability to extinguish conditioned fear (Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). Intuitively, increased exploration in a new environment seems to contradict susceptibility to helplessness. However, it is consistent with a predisposition to PTSD, given the association of this disorder with increased novelty seeking, as measured by the Cloninger Tridimensional Personality Questionnaire (Richman & Frueh, 1997; Wang *et al.*, 1997).

Prior to this work, no studies have examined the predictive value of all three behavioral dimensions in the development of helplessness. Based on data from the congenitally helpless strain, I hypothesized that increased exploration in response to novelty, reduced consumption of a rewarding sucrose solution and enhanced passive avoidance of a harmful stimulus would predict helpless behavior (defined by high escape latencies) in a stress-susceptible rat strain.

1.4 PHYSIOLOGIC CORRELATES OF HELPLESSNESS: HYPOTHALAMIC-PITUITARY ADRENAL AXIS AND AUTONOMIC NERVOUS SYSTEM

The stress response involves both the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (Figure 1.1) and it has been postulated that a dysregulation in the stress pathways might play a role in the pathogenesis of depression (Brown *et al.*, 2009; Jemerin & Boyce, 1990; Pariante & Lightman, 2008; Pariante, 2009). For example, a major finding among depressed individuals has been increased levels of glucocorticoid hormones- the main product of the HPA axis (Pariante & Lightman, 2008; Pariante, 2009) and an altered autonomic tone has also been associated with depression and anxiety (Sheps & Sheffield, 2001). Furthermore, a dissociated HPA axis was observed in congenitally helpless rats characterized by a

hyperactive paraventricular hypothalamus (Shumake *et al.*, 2001) and reduced plasma corticosterone both at baseline (Edwards *et al.*, 1999) and in response to stress (Edwards *et al.*, 2000; King & Edwards, 1999).

In addition, parasympathetic vagus nerve fibers control heart rate variability, and an elevated vagal tone may provide protection against malignant cardiac arrhythmias (Friedman & Thayer, 1998). Interestingly, depressed subjects showed diminished heart rate variability compared to non-depressed patients and reduced heart rate variability has been linked to a poorer prognosis in patients with coronary artery disease (Sheps & Sheffield, 2001). Negative mood states have also been linked to autonomic nervous system disturbances and cardiac events (Schwarz *et al.*, 2003). Moreover, there are individual differences in the responsiveness of the stress pathways in both animals and humans (Jemerin & Boyce, 1990; Levine, 1975). For instance, infants who displayed greater overt anxiety during maternal separation and exposure to novelty had significantly higher urinary cortisol excretion during the episodes (Tennes *et al.*, 1977) and adult rats that were not handled by experimenters as neonates had less effective negative feedback regulation of adrenocorticotropic hormone (ACTH), resulting in greater corticosterone secretion during stress (Jemerin & Boyce, 1990; Meaney *et al.*, 1988).

Furthermore, reasons to study physiologic parameters such as heart rate and heart rate variability as an index of biobehavioral responsiveness to the environment include: 1) they can be measured non-invasively, 2) variations in heart rate and blood pressure reflect activity and responsivity of the autonomic nervous system and provide a measure of the functional state of central neurobiological regulatory mechanisms, including the limbic system and hypothalamus and 3) individual differences in the physiological responses to stress are linked to depression and anxiety (Brown *et al.*,

2009; Jemerin & Boyce, 1990; Marchei *et al.*, 2009; Sheps & Sheffield, 2001) . Temperament or personality is also linked to physiologic parameters such as heart rate and cortisol. For example, novelty seeking was associated with lower heart rate among men and women (Puttonen *et al.*, 2008), and was inversely correlated with resting stress hormone concentrations (i.e. cortisol) (Tyrka *et al.*, 2007). Furthermore, among PTSD combat veterans, increased novelty-seeking was inversely correlated with cortisol levels (Wang *et al.*, 1997). This dissertation also aimed to identify individual differences in the physiological response to stress, including heart rate, heart rate variability and plasma corticosterone that could predict helpless behavior among Holtzman rats.

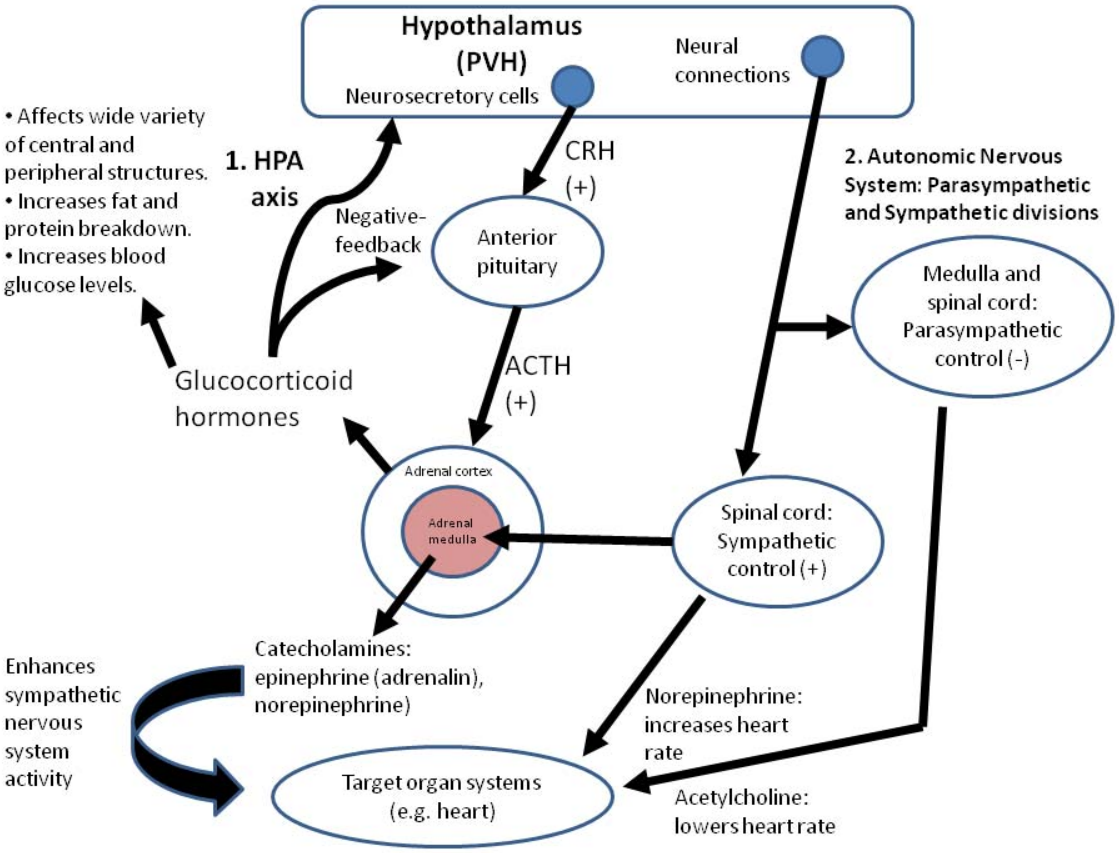

Figure 1.1 Stress response pathways. The hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system are critical mediators of the stress response. 1) Neurons in the paraventricular nucleus of the hypothalamus (PVH) secrete corticotropin releasing hormone (CRH), which stimulates synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids can also exert negative-feedback effects and inhibit ACTH and CRH release. Glucocorticoids affect a wide variety of systems including the hypothalamus and other emotion-associated areas of the brain. 2) The PVH also contains neurons that innervate both the parasympathetic and sympathetic neurons in the medulla and spinal cord, thus playing a major role in regulating the autonomic nervous system (ANS). Most target organs have dual innervation of the sympathetic and parasympathetic divisions of the ANS. In general, activity of the parasympathetic division is consistent with resting conditions during which eating, digestion, defecation, and other vegetative functions are emphasized and the sympathetic system decreases the activity of organs not essential for the maintenance of physical activity and shunts blood and nutrients to structures that are active during physical exercise. This is sometimes referred to as the fight-or-flight response (Seeley *et al.*, 1998a).

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1.5 TRANSLATIONAL RELEVANCE OF A STRESS-SUSCEPTIBLE ANIMAL MODEL TO EXAMINE ANTIDEPRESSANT TREATMENT RESPONSE

In 2005, antidepressants surpassed antihypertensive agents to become the most commonly prescribed class of medications in office-based and hospital outpatient–based medical practice (Olfson & Marcus, 2009). Selective serotonin reuptake inhibitor (SSRI) antidepressants, of which fluoxetine is the prototypical drug, are commonly used to treat depression and anxiety disorders such as post-traumatic stress disorder (PTSD) (Devane *et al.*, 2005; Hemels *et al.*, 2002; Olfson & Marcus, 2009). However, it is estimated that 40% of patients taking SSRI's do not attain relief from depression following 6- 8 weeks of therapy (Debattista & Solvason, 2003; Rosenzweig-Lipson *et al.*, 2007; Thase *et al.*, 2001). Even more disappointing is that these compounds may only be partially effective with 25 to 45% of patients on antidepressants becoming symptom-free and/or reaching remission (Thase *et al.*, 2001). This low response rate may be due to the fact that not much is known regarding the neural mechanisms that underlie treatment response. SSRI drugs are thought to relieve symptoms by blocking neuronal serotonin re-uptake and consequently increasing post-synaptic levels of serotonin (American Society of Health-System Pharmacists, 2001). This mechanism of action supports the biogenic amine deficiency hypothesis of mood disorders (Markus, 2008; Syvalahti, 1987; Van der Does, 2001; Sachar & Baron, 1979). However, peak re-uptake inhibition can occur within 3 to 8 hours of SSRI administration (American Society of Health-System Pharmacists, 2001) and this effect does not correlate with the observed therapeutic response which can take approximately 4 to 8 weeks in humans (Keller, 2005).

Furthermore, drugs that increase brain concentrations of serotonin and/or norepinephrine have also been used in patients that fail to respond to SSRI's but there

are still populations of patients that do not adequately respond to this treatment (Hirschfeld *et al.*, 2002). Based on the previous findings, it is highly unlikely that a deficit of monoamines, such as serotonin and/or norepinephrine, is the sole cause of complex behavioral disorders involving distributed functions of the brain, such as emotions and mood. The pathophysiology of stress-related disorders is more likely mediated by a dysfunction in diverse neural networks and thus, a broader approach should be applied to understand the network mechanisms underlying treatment response. Notably, Mayberg et al., (2000) reported that reciprocal changes in patterns of activity in the prefrontal cortex and subgenual cingulate (known as infralimbic cortex in rodents) predicted a response to fluoxetine in depressed patients. Increased knowledge of the inter-regional functional connections that are differentially affected in treatment responders and non-responders could aid in the understanding of neural network mechanisms underlying treatment response.

In addition, most animal studies examining the effects of antidepressants have used a “normal” population without *a priori* knowledge of susceptibility to depressive-like behavior (Cryan *et al.*, 2005a; Detke *et al.*, 1997; Porsolt *et al.*, 1978). However, antidepressants are not usually administered to humans that do not have a therapeutic necessity and/or valid diagnosis. Furthermore, not all brains are similar and although we assume that a drug, like an antidepressant, will cause the same effect in every individual, this is probably not the case as neural systems display variability in their homeostatic strategies (Goaillard *et al.*, 2009). These effects are thus expected to be different between rat strains with diverse susceptibilities to helplessness. The use of a vulnerable strain could be of immense value to examine the neural mechanisms underlying response to antidepressant treatment. The present work identified the

network mechanisms underlying antidepressant treatment response using a stress-susceptible rat strain.

1.6 CYTOCHROME OXIDASE IS A LONG-TERM MARKER OF BRAIN METABOLIC CAPACITY

In humans, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have been used to examine neural correlates of depression/ PTSD and the effects of antidepressants (Drevets *et al.*, 1997; Drevets, 2000; Hull, 2002; Mayberg *et al.*, 1999; Mayberg *et al.*, 2000; Rauch *et al.*, 2006; Rosier *et al.*, 2009; Seminowicz *et al.*, 2004; Shin *et al.*, 2004; Videbech, 2000). However, it is difficult to obtain optimal resolution of small subcortical structures, which can also be part of a more complete functional network. Metabolic mapping techniques in animals are useful tools for studying the functional effects because they can provide a common brain measure for investigating apparent behavioral similarities between animals and humans. In addition to having access to the entire brain in rats, these techniques also provide the spatial resolution to include individual subcortical nuclei. An example of such a technique is quantitative cytochrome oxidase (CO) histochemistry. CO is the terminal respiratory enzyme in the mitochondrial electron transport chain that is critical for glucose oxidation and the production of adenosine triphosphate (ATP) (Figure 1.2). As such, it serves as an endogenous marker of neuronal metabolic activity (Wong-Riley, 1989). Furthermore, changes in metabolic activity primarily reflect metabolic capacity resulting from up or down-regulation of brain cytochrome oxidase which requires protein synthesis and axonal transport. Therefore, CO is a long-term indicator of brain metabolic capacity (Wong-Riley *et al.*, 1998) making histochemical quantification of cytochrome oxidase activity an ideal marker for examining the long-lasting effects of antidepressants on brain metabolism.

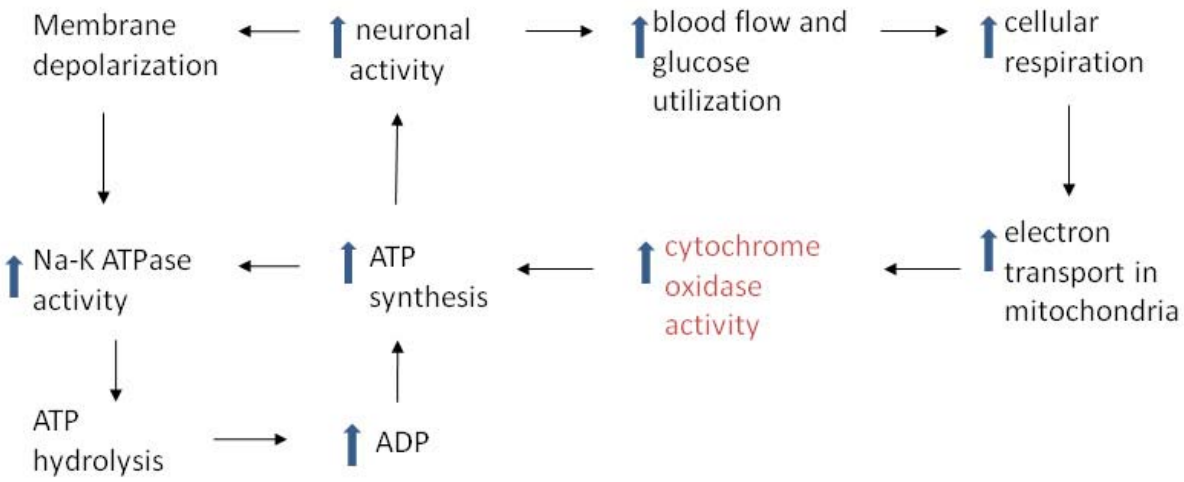

Figure 1.2 Schematic diagram of the relationship between neuronal activity and energy metabolism. Energy consumed by active ion transport is generated by oxidative metabolism and cytochrome oxidase is a critical component of this process (Wong-Riley, 1989).

Cytochrome oxidase has been demonstrated to be a useful tool for mapping metabolic changes associated with the helpless phenotype. For instance, congenitally helpless rats show cytochrome oxidase activity differences in frontal and cingulate regions comparable to those affected in human depression, with directional changes identical to those seen in human neuroimaging studies of glucose metabolism and cerebral blood flow (Baxter, Jr. *et al.*, 1989; Bench *et al.*, 1993; Drevets *et al.*, 1997; Drevets, 2000; Liotti & Mayberg, 2001; Shumake *et al.*, 2000). Congenitally helpless rats showed a pattern of reduced metabolism in dorsal frontal, medial orbitofrontal, and anterior cingulate cortex accompanied by no change in metabolism in medial frontal (prelimbic) cortex and elevated metabolism in infralimbic cortex (homologous to human subgenual cingulate cortex).

While several PET studies have used deoxyglucose radiotracers to study the effects of depression and treatment, cytochrome oxidase has never been measured in depressed humans. The question then arises, why use cytochrome oxidase histochemistry instead of deoxyglucose autoradiography to examine the effects of helplessness and response to treatment? First, the goals of CO and deoxyglucose experiments are different. The CO technique is suited for assessing the effects of chronic training or manipulations over days or weeks on the alterations of activity levels linked to the sustained metabolic demands on the brain regions involved, while studies with deoxyglucose assess short-term changes in metabolic activity evoked by stimulation during the post-injection survival period of the radiotracer (Gonzalez-Lima & Cada, 1998). As such, there is not a linear relationship between a region's deoxyglucose uptake and its cytochrome oxidase activity (Gonzalez-Lima & Cada, 1998), but both provide a measure of oxidative metabolism. Cytochrome oxidase is unique in that it marks cumulative, long-term neuronal activity, while deoxyglucose uptake reflects state-

dependent activity spanning approximately 45 minutes following tracer injection (Gonzalez-Lima & Cada, 1998). In other words, cytochrome oxidase reflects stabilized differences or “static” changes in neural activity (Gonzalez-Lima & Sadile, 2000; Papa *et al.*, 1998) as opposed to “dynamic” differences in mean glucose levels as revealed by deoxyglucose autoradiography. Furthermore, “resting state” cerebral glucose metabolism as measured in human PET studies probably assimilates metabolic capacity as determined by CO histochemistry in animals. In human studies, subjects are instructed on the experimental procedures and the imaging likely takes place in a stress free environment, whereas rodents are removed from their home cages and usually restrained and subjected to a certain amount of stress before the collection of brain tissue. These stressful manipulations can affect short-term glucose uptake and brain activity. The main goal of this project was to determine the neurobiological predisposition to develop helpless behavior and the long term effects of antidepressant treatment —not the brain’s evoked response to stress. Thus, quantitative cytochrome oxidase histochemistry provides a unique way to isolate this innate component by identifying the brain regions affected in helplessness and chronic antidepressant treatment.

1.7 CORTICAL AND SUBCORTICAL EFFECTS OF ANTIDEPRESSANTS

Although antidepressant treatment effects are highly variable in the human imaging literature, studies using SSRIs have most often found increased cerebral glucose metabolism in prefrontal and anterior cingulate cortex and decreases in the hippocampus and in ventromedial frontal, subgenual cingulate, and anterior insular cortex (Brody *et al.*, 1999; Buchsbaum *et al.*, 1997; Mayberg *et al.*, 2000). Importantly, the only brain changes reliably associated with depression remission have been an increase in prefrontal cortex and dorsal anterior cingulate metabolism and a decrease of subgenual cingulate metabolism compared to baseline levels (Mayberg *et al.*, 1999).

This pattern of opposite effects is consistent with a model put forth by Mayberg (1997), who has suggested that there is a ventral-dorsal, limbic-cortical opposition in depression, as well as in normal sadness. I hypothesized that characterization of the network effects of antidepressant treatment response using cytochrome oxidase histochemistry in a stress-susceptible rat strain would show a similar limbic-cortical pattern.

Moreover, identification of the subcortical effects of antidepressants could represent additional predictors of treatment response. For instance, pharmacologic treatments are viewed as acting through “bottom-up” or combined “bottom-up” and “top-down” mechanisms, since brain stem nuclei —most notably dorsal raphe and locus ceruleus —are major sites of action for most antidepressants, with secondary effects mediated in remote cortical sites through afferent projections and post-synaptic receptor mechanisms (Chaput *et al.*, 1991; Hyman & Nestler, 1996). Furthermore, a fluorodeoxyglucose-PET study examined the involvement of cortical (lateral prefrontal, medial frontal and anterior cingulate cortex), limbic (subgenual cingulate, orbital frontal cortex) and subcortical regions (anterior thalamus and hippocampus) in response to antidepressant medication (Seminowicz *et al.*, 2004). In this study, non-responders were characterized by greater involvement of a limbic-subcortical network and absence of cortical-cortical and cortical-limbic interactions.

Using animal models, prior knowledge of the subcortical nuclei that can potentially be affected in depression and treatment response can help guide clinical expectations with the advent of newer, more advanced human neuroimaging techniques that have greater resolution. For example, Morris et al. (1999) used a high-sensitivity positron emission tomography (PET) scanner to acquire images on several subcortical regions. The investigators induced transient depressive relapses in volunteer patients by rapidly depleting plasma tryptophan, the precursor of serotonin (5-HT) and measured

regional cerebral blood flow. Neural activity in several 5-HT-related brain areas, e.g., dorsal raphe, habenula, septal region, amygdala, and orbitofrontal cortex, covaried significantly with plasma levels of tryptophan and ratings of depressed mood (Morris *et al.*, 1999). Another human study used functional magnetic resonance imaging and found similar effects in the habenula after serotonin depletion (Rosier *et al.*, 2009). These studies highlight that the psychopharmacology of mood disorders involve cortical and subcortical interactions. Animal models can be useful in understanding these interactions and providing a complete functional map of the effects of antidepressants on the brain.

1.8 FUNCTIONAL AND ANATOMICAL CONNECTIVITY: STRUCTURAL EQUATION MODELING

The term connectivity refers to both anatomical and functional communication between two or more brain regions. Anatomical connectivity is defined as a structural link between brain regions, whereas functional connectivity is defined as neurophysiological covariance between brain regions. The use of covariance for determining functional relationships has a long history in neuroscience, beginning with multiunit microelectrode recording (Gerstein *et al.*, 1978) and metabolic brain mapping with fluorodeoxyglucose (FDG) (Horwitz *et al.*, 1984). Regions which share higher covariance (across participants or across time within a participant) are assumed to share a functional connection, regardless of whether these regions share an anatomical connection. That is, direct anatomical connectivity is neither necessary nor sufficient for the existence of functional connectivity. It is not necessary because a functional connection can arise between two regions in the absence of a direct anatomical connection, for example when both regions share anatomical connectivity with a third, mediating region. It is not sufficient because anatomical connections provide only the

potential for functional connections. In theory, the actual covariance between two regions depends on how much information they exchange, which can increase or decrease depending on the specific behavioral challenge.

One way of integrating functional and anatomical connectivity is with structural equation modeling (SEM) or path analysis. SEM is a multivariate modeling technique first applied to investigate brain network interactions by McIntosh and Gonzalez-Lima (1991, 1992, 1993, 1994, and 1995). SEM has been used to analyze neural network interactions using FDG, fMRI and cytochrome oxidase data (Bruchey *et al.*, 2007; Buchel & Friston, 1997; McIntosh & Gonzalez-Lima, 1991; McIntosh & Gonzalez-Lima, 1992; McIntosh & Gonzalez-Lima, 1993; McIntosh & Gonzalez-Lima, 1994; McIntosh & Gonzalez-Lima, 1995; Puga *et al.*, 2007; Seminowicz *et al.*, 2004). Wright (1934) first developed the path analysis method to study the functional relationships between *stable* variables such as genetic traits manifested as phenotypic features like skin color (Wright, 1934). Using cytochrome oxidase activity data, the SEM approach is suited for analysis of *stable* network interactions because cytochrome oxidase histochemistry maps the cumulative endpoint of long-term neuronal enzymatic activity (Gonzalez-Lima & Cada, 1998).

Interpretations of the correlational analysis of metabolic mapping data can become complicated when one wants to extend the discussion beyond pairwise covariance relationships (McIntosh & Gonzalez-Lima, 1993). When applied to neural networks, SEM uses information about known anatomical pathways and correlation coefficients of activity between brain regions to identify the functional pathways in a given experiment. This computational method can be used to simultaneously quantify the directional influences or effective connectivity between many brain areas and allows

for the assessment of changes in entire systems. This technique was applied in the study of the network effects of antidepressant treatment response.

1.9 NEURAL MECHANISMS UNDERLYING SUSCEPTIBILITY TO HELPLESSNESS

To further support the use of the Holtzman strain as a potential animal model for studying stress-induced psychopathologies, we needed to characterize not only their behavioral susceptibility and response to antidepressant treatment, but also the neural correlates of helpless behavior. Human neuroimaging studies have provided much information on neural networks that underlie mental disorders such as depression (Drevets, 2000; Mayberg, 1997; Mayberg *et al.*, 1999) and PTSD (Rauch *et al.*, 2006; Shin *et al.*, 2004). In general the dorsal lateral prefrontal cortex and ventral limbic and paralimbic structures (orbitofrontal and insular cortex) seem to be the most universally affected (Mayberg *et al.*, 1999; Molina *et al.*, 2007). In addition, studies using the congenitally helpless rat model and metabolic brain mapping with cytochrome oxidase have provided much insight into the functional neuroanatomy of helplessness vulnerability (Shumake *et al.*, 2000; Shumake & Gonzalez-Lima, 2003).

1.9.1 Candidate brain regions suggested from human and animal studies

Humans. It is likely that many brain regions mediate the diverse symptoms of depression. This is supported by human neuroimaging studies which have demonstrated changes in blood flow and metabolism in several regions including the prefrontal-cingulate cortex, hippocampus, basal ganglia, amygdala, thalamus, habenula and dorsal raphe (Drevets, 2000; Liotti & Mayberg, 2001; Morris *et al.*, 1999; Nestler *et al.*, 2002; Rosier *et al.*, 2009). These findings will be discussed in detail in the following subsections.

Fewer neuroimaging studies have been conducted on post-traumatic stress disorder compared to depression. Most PTSD studies have implicated the prefrontal-cingulate cortex, hippocampus, amygdala, and other limbic and paralimbic structures but the direction of the changes is not always consistent (Hull, 2002). This could be due to differences in the methods used among the studies. For example, examining activity during symptom provocation versus baseline conditions, analysis among different types of trauma victims (combat veterans versus survivors of childhood physical or sexual abuse), and the presence of different psychotropic medications and comorbidities such as substance abuse and depression (Bonne *et al.*, 2003; Hull, 2002). Symptom provocation studies are the most commonly performed. These studies report increased activity in the premotor frontal region and decreased activity in the anterior cingulate and middle temporal cortex (Bremner *et al.*, 1999; Lanius *et al.*, 2001; Rauch *et al.*, 1996; Shin *et al.*, 1999; Shin *et al.*, 2001). Furthermore, a baseline resting state study reported that reduced cortisol signaling predicted increased hippocampal and decreased frontal-cingulate blood flow in subjects susceptible to PTSD (Bonne *et al.*, 2003). Structural MRI studies revealed reduced hippocampal volume in PTSD patients (Bremner *et al.*, 1997b; Gurvits *et al.*, 1996; Stein *et al.*, 1997; Villarreal *et al.*, 2002). Notably, Gilbertson et al. (2002) found decreased hippocampal volume in PTSD patients' monozygotic twins who never experienced trauma, suggesting that this may be a marker of vulnerability (Gilbertson *et al.*, 2002).

Animals. Congenitally helpless rats showed hypermetabolism in the habenula, interpeduncular nucleus, paraventricular hypothalamus, hippocampus and infralimbic cortex, and they had decreased metabolism in the dorsomedial prefrontal cortex, basal ganglia, amygdala, septum and ventral tegmental area (Shumake *et al.*, 2000; Shumake *et al.*, 2001; Shumake *et al.*, 2002; Shumake & Gonzalez-Lima, 2003; Shumake *et al.*,

2003). Furthermore, learned helplessness studies in animals have implicated the hippocampus (Lachman *et al.*, 1993; Leshner & Segal, 1979; Papolos *et al.*, 1993), bed nucleus of stria terminalis (Hammack *et al.*, 2004), septum (Steciuk *et al.*, 1999), habenula (Amat *et al.*, 2001) and dorsal raphe nucleus (Grahn *et al.*, 1999; Grahn *et al.*, 2000; Grahn *et al.*, 2002; Hammack *et al.*, 2002; Maier *et al.*, 1993; Maier *et al.*, 1994; Maier *et al.*, 1995) in the effects of acquiring LH after exposure to inescapable shock.

The following subsections will highlight important findings in several brain regions mentioned above. In addition, comparisons will be made between observations in humans and learned helplessness studies with rats. Similar effects were expected in the Holtzman rat strain. Understanding the neural mechanisms underlying helplessness susceptibility can be used to support the use of the Holtzman strain as a model of stress-related psychopathology.

Table 1.2 Candidate brain systems suggested from human depression and animal studies

Systems	Human	Congenitally Helpless*	Learned helplessness
Prefrontal cortex	↓	↓	?
Subgenual cingulate/ infralimbic cortex	↑	↑	?
Orbital-insular cortex	↑or ↓	↓	?
Hypothalamic-pituitary-adrenal axis	↑	↑ PVH with ↓ corticosterone levels	↑ corticosterone with dexamethasone challenge vs. naïve and NH rats
Hippocampus	↓ volume	↑	↑ glutamate and ↓ GABA activity relative to NH rats
Septum	?	↓	↓ 5HT levels, ↑ GABA A receptors compared to naïve controls
Extended amygdala	↑ or no change	↓	Maybe ↑, lesions reduce immobility or prevent LH after inescapable shock
Basal ganglia	↓	↓	↓ D2 receptors in caudate relative to naïve and NH
Mesolimbic Pathway VTA → Accumbens	Maybe ↓, anhedonia is core symptom in depression	↓	↓ D1 receptor in accumbens vs. NH rats
Dorsal raphe	?	No change	↑ 5HT receptor activity in IS vs. yoked-ES rats, and ↑ 5HT release correlated with helpless behavior
Habenula	↑	↑	Probably ↑, lesions prevent LH
Interpeduncular nucleus	Receives large Ach input, evidence of ↑ cholinergic activity in depression	↑	?

Arrows indicate increase or decrease in activity as measured by blood flow or metabolism, unless otherwise indicated. PVH (paraventricular hypothalamic nucleus), VTA (ventral tegmental area), 5HT (serotonin), GABA (gamma-aminobutyric acid), NH (non-helpless), IS (inescapable shock), ES (escapable shock). * Compared to congenitally non-helpless rats.

1.9.1.1 Prefrontal-cingulate cortex

Depression occurs commonly after strokes that affect the prefrontal cortex (PFC) (Morris *et al.*, 1996) and structural MRI has shown that depression is associated with reduced PFC volume (Coffey *et al.*, 1993). Furthermore, in the human neuroimaging literature, abnormally decreased blood flow and metabolism in dorsolateral prefrontal areas are extensively replicated findings in depression (Baxter, Jr. *et al.*, 1989; Bench *et al.*, 1992; Bench *et al.*, 1993; Drevets, 2000; Mayberg, 2003; Videbech, 2000). In fact, Mayberg et al. (1997) proposed prefrontal limbic-cortical dysregulation as a model for depression characterized by dorsolateral hypo-functioning with overactive ventral limbic areas including the subgenual cingulate.

The anterior cingulate cortex situated anterior and ventral to the genu of the corpus callosum is termed the pregenual and subgenual cingulate, respectively. In the subgenual cingulate, known as the infralimbic cortex in rodents (Shumake & Gonzalez-Lima, 2003), metabolism is increased in depressed patients relative to healthy subjects after correcting for volumetric reductions in gray matter (Drevets *et al.*, 1997; Drevets, 2000). This is consistent with the observation that effective antidepressant drug treatment can decrease metabolic activity in this region among patients with major depression (Buchsbaum *et al.*, 1997; Drevets, 1999; Mayberg *et al.*, 2000) and that in healthy subjects, cerebral blood flow increases in the subgenual cingulate during sadness induction (George *et al.*, 1995).

The pregenual cingulate —homologous to the rat prelimbic cortex —has shown both elevated and suppressed blood flow and metabolism in untreated depression (Bench *et al.*, 1993; Drevets *et al.*, 1992; Drevets & Raichle, 1998; Mayberg, 1994; Mayberg *et al.*, 1997). Notably, elevated activity in the pregenual anterior cingulate predicts that a patient will respond well to antidepressant treatment, while suppressed

activity predicts that a patient will show a poor response (Mayberg *et al.*, 1997). In contrast, Brody et al. (1999) found that lower metabolism in this region predicted superior response to paroxetine (SSRI antidepressant) (Brody *et al.*, 1999). The extent to which these discrepancies could be explained by differential effects in subregions of this area, clinical demographics, or type of antidepressant treatment used in the studies (tricyclic antidepressants, bupropion, and/or SSRI's) are unknown.

1.9.1.2 Orbital and anterior insular prefrontal cortex

The orbital and the anterior insular cortex show abnormally increased blood flow and metabolism in unmedicated subjects with depression (Baxter, Jr. *et al.*, 1987; Biver *et al.*, 1994; Cohen *et al.*, 1992; Drevets *et al.*, 1992; Ebert *et al.*, 1991). Blood flow and metabolism also increase in these areas during induction of sadness and anxiety in healthy subjects, and during induced anxiety and obsessive states in patients with obsessive-compulsive, post-traumatic stress, simple phobia, and panic disorders (Drevets & Raichle, 1998; Shin *et al.*, 1999). However, although metabolic activity is increased in out-patient treatment responsive patients, more severely-ill or treatment refractory patients either did not show differences or had decreased metabolism relative to control subjects (Drevets *et al.*, 1997; Ketter *et al.*, 1999; Mayberg *et al.*, 1997). Drevets et al. (2000) argued that enhanced orbital activation may represent an endogenous attempt to attenuate emotional expression and interrupt perseverative patterns of aversive thoughts but that reduced orbital metabolism may reflect a primary pathology related to monoamine dysfunction. For example, orbital cortex metabolism is decreased in depressed patients with Parkinson's disease, relative to non-depressed subjects, suggesting that dopamine depletion may impair orbital cortex function. Furthermore, remitted patients with depression who relapsed under serotonin depletion had reduced metabolism restricted to the medial portion of the orbital-frontal cortex

(Bremner *et al.*, 1997a), a region which was also hypometabolic in the congenitally helpless rat (Shumake *et al.*, 2000).

1.9.1.3 Hypothalamic pituitary adrenal (HPA) axis

A prominent mechanism by which the brain reacts to acute and chronic stress is activation of the hypothalamic pituitary adrenal (HPA) axis (Iversen *et al.*, 2000a; Seeley *et al.*, 1998b). Excessive activation of the HPA axis is observed in approximately half of individuals with depression and these abnormalities can often be normalized with antidepressant treatment (Holsboer, 2001; Nemeroff & Vale, 2005; Pariante & Lightman, 2008; Sachar & Baron, 1979; Nestler *et al.*, 2002; Arborelius *et al.*, 1999). In addition, some patients exhibit increased baseline levels of plasma cortisol and ACTH, and are unable to adequately suppress plasma cortisol and ACTH when challenged with dexamethasone, a synthetic glucocorticoid (Holsboer *et al.*, 1982; Nestler *et al.*, 2002). Increased CRH secretion has been observed among depressed individuals (Arborelius *et al.*, 1999; Holsboer, 2001; Kasckow *et al.*, 2001) and postmortem studies have shown elevated levels of CRH expressing neurons in the PVH (Raadsheer *et al.*, 1994). Furthermore, congenitally helpless rats have a dissociated HPA axis characterized by a hypermetabolic PVH (Shumake *et al.*, 2001), ACTH hypersecretion in response to stress (King & Edwards, 1999) and low levels of corticosterone production both at baseline (Edwards *et al.*, 1999) and in response to stress (Edwards *et al.*, 2000; King *et al.*, 1993; King & Edwards, 1999). In addition, rats that became helpless after inescapable shock had decreased ability to suppress corticosterone levels after dexamethasone challenge (Greenberg *et al.*, 1989).

Glucocorticoids can dramatically affect behavior and neurophysiologic function via direct actions on numerous brain regions including the hypothalamus, hippocampus and other emotion-associated brain areas (Iversen *et al.*, 2000b; Nestler *et al.*, 2002;

Seeley *et al.*, 1998b; Birmingham *et al.*, 1993). Therefore, a dysfunction in this system can mediate many of the effects seen in depression. This is further supported by the striking parallels among the stress response, severe depression, and centrally administered CRH in physiologic aspects such as increased arousal, decreased appetite, decreased sexual behavior, and increased heart rate and blood pressure (Arborelius *et al.*, 1999; Holsboer, 2001).

1.9.1.4 Hippocampus

The hippocampus can influence the activity of hypothalamic CRH containing neurons via inhibitory inputs; and glucocorticoids can regulate hippocampal and PVH neurons, by exerting feedback effects on the HPA axis (Nestler *et al.*, 2002; Pariante & Lightman, 2008). Sustained elevations of glucocorticoids, as seen under conditions of prolonged or severe stress, may damage hippocampal neurons. This damage may involve a reduction of dendritic branching and a loss of specialized dendritic spines (McEwen, 2000; Sapolsky, 2000). Loss of hippocampal function is expected to reduce its inhibitory control of the HPA axis, which would further increase circulating glucocorticoid levels and subsequent hippocampal damage. Hippocampal volume reductions are reported in several magnetic resonance imaging (MRI) studies of patients with depression (Bremner *et al.*, 2000; Krishnan *et al.*, 1991; Sapolsky, 2000; Shah *et al.*, 1998; Sheline *et al.*, 1996). However, hippocampal function has rarely been directly assessed with functional neuroimaging. The only two studies found in the literature report opposite findings. A PET study reported reduced left hippocampal metabolism, which inversely correlated with depression severity (Saxena *et al.*, 2001). However, a study using fMRI reported that hippocampal activity did not differ between depressed patients and control participants during encoding or retrieval of an associative learning paradigm (Werner *et al.*, 2009).

However, congenitally helpless rats showed hypermetabolism in the hippocampus (Shumake *et al.*, 2002) which is consistent with the finding of increased glutamate activity and decreased GABA activity in the hippocampi of learned helpless rats (Petty & Sherman, 1981). Hippocampal hypermetabolism may be related to functional changes in processing of glucocorticoid feedback. For instance, low levels of corticosterone secretion in the congenitally helpless rat may lead to compensatory upregulation of glucocorticoid receptors in the hippocampus. Moreover, chronic antagonism of hippocampal glucocorticoid receptors (which can lead to a similar compensatory mechanism) increases vulnerability to helplessness (Papolos *et al.*, 1993) and these receptors are dysfunctional in congenitally helpless rats (Lachman *et al.*, 1993). Even though the literature has mainly implicated reduced hippocampal volume (Bremner *et al.*, 2000; Sheline *et al.*, 1996) and decreased hippocampal neurogenesis in the effects of depression (Elder *et al.*, 2006; Paizanis *et al.*, 2007), elevated hippocampal metabolism is consistent with some human neuroimaging data, which has shown increased metabolism in the hippocampus of PTSD patients with low cortisol levels (Bonne *et al.*, 2003).

1.9.1.5 Septum

The hippocampus may regulate PVH activity via its projections to the lateral septal nucleus and bed nucleus of the stria terminalis (Cullinan *et al.*, 1993; Sheehan & Numan, 2000), which are the only forebrain regions with significant projections to the PVH (Silverman *et al.*, 1981). In turn, the septum may influence the hippocampus through cholinergic and noncholinergic projections from the medial septal nucleus and the nucleus of the diagonal band (Amaral & Kurz, 1985). Furthermore, the septal area has been directly implicated in helpless behavior. Sherman and Petty (1980) found that learned helplessness was associated with decreased septal serotonin (5-HT) release

and that 5-HT microinjected into the lateral septum reversed learned helpless behavior (Sherman & Petty, 1980). They also found that systemic administration of the antidepressant desipramine reversed helpless behavior while, in parallel, normalizing septal 5-HT. Rats that acquired learned helplessness also had increased densities of septal GABA receptors (Kram *et al.*, 2000). This is consistent with hypometabolism in the septum of congenitally helpless rats compared to congenitally non-helpless rats (Shumake *et al.*, 2002).

1.9.1.6 Amygdala and bed nucleus of stria terminalis

The amygdala has mainly been studied for its role in conditioned fear (Cahill *et al.*, 1999; Davis, 1998; LeDoux, 2000) but it is also equally important for conditioned responses to rewarding stimuli, including drugs of abuse and natural rewards (Everitt *et al.*, 1999). In fact, some researchers view the amygdala as part of a larger circuit — termed the extended amygdala —which includes the nucleus accumbens and the bed nucleus of stria terminalis (de Olmos & Heimer, 1999). The amygdala and related structures have been implicated in anxiety, PTSD, drug addiction and depression (Nestler *et al.*, 2002). For example, elevations of resting regional cerebral blood flow or glucose metabolism in the amygdala have been reported in depressed patients (Ho *et al.*, 1996; Nofzinger *et al.*, 1999; Drevets *et al.*, 1992). Furthermore, metabolic rate in the amygdala correlated with depression severity (Drevets *et al.*, 1992) or negative affect (Abercrombie *et al.*, 1998) in depressed patients. However, amygdala changes have not been observed in most studies and may only be present in patients with high levels of dispositional negative affect and anxiety (Davidson *et al.*, 2002).

Elevated amygdala activity may play a role in helpless behavior since lesions of the basolateral and central nuclei reduce freezing or immobility following inescapable shock. However, these lesions did not prevent the escape deficit (Maier *et al.*, 1993), but

lesions of the bed nucleus of stria terminalis did prevent the escape deficit that normally follow inescapable shock (Hammack *et al.*, 2004). In contrast, the congenitally helpless rat showed hypometabolism in the basolateral and central amygdala nuclei (Shumake & Gonzalez-Lima, 2003), which is consistent with studies that reported dopamine depletion in the basal and central amygdala of depressed humans (Klimek *et al.*, 2002). It is purported that at rest, amygdala hypometabolism is reflective of an impaired reward system, but that it could become hyperactive under conditions of stress, reflecting enhanced fear and anxiety (Shumake & Gonzalez-Lima, 2003).

1.9.1.7 Basal Ganglia

Human neuroimaging studies have reported reduced blood flow or metabolism in the basal ganglia of depressed patients (Baxter, Jr. *et al.*, 1985; Drevets *et al.*, 1992; Rogers *et al.*, 1998; Videbech, 2000). Structural imaging studies and postmortem analysis of depressed individuals revealed reduced volume of the caudate, putamen and ventral striatum (Baumann *et al.*, 1999; Krishnan *et al.*, 1992; Parashos *et al.*, 1998). Similarly, congenitally helpless rats show decreased cytochrome oxidase activity throughout the basal ganglia including the caudate putamen, globus pallidus, ventral pallidum and nucleus accumbens (Shumake & Gonzalez-Lima, 2003). Rats that acquire learned helplessness after exposure to inescapable stress also showed less dopamine receptor densities in regions of the basal ganglia (Kram *et al.*, 2002).

Dopaminergic transmission dysfunction in the basal ganglia has been implicated in anhedonia defined as the inability to experience pleasure and psychomotor retardation (the slowing of physical, mental, and emotional reactions) —both symptoms of major depression (American Psychiatric Association, 2000b; Nestler *et al.*, 2002). Furthermore, depression often accompanies and precedes the motor signs of Parkinson's disease, a neurodegenerative disorder characterized by dopaminergic cell

loss in the basal ganglia (Mayeux *et al.*, 1981; Rodriguez-Oroz *et al.*, 2009; Stern & Langston, 1985).

1.9.1.8 Mesolimbic dopaminergic pathway: ventral tegmental area and nucleus accumbens

The nucleus accumbens is a target of the mesolimbic dopamine system, which arises from dopamine neurons in the ventral tegmental area (VTA) of the midbrain (Kupfermann *et al.*, 2000). VTA neurons also innervate other limbic structures including the amygdala, septum and limbic cortex, especially the anterior cingulate cortex (Berger *et al.*, 1992; Oades & Halliday, 1987). The VTA to accumbens pathway plays a critical role in reward (Nestler & Carlezon, Jr., 2006); however, despite the fact that anhedonia is one of the core symptoms of depression and PTSD (American Psychiatric Association, 2000b), not much is known regarding the role of this pathway in the etiology of mood disorders. Serotonin and norepinephrine mechanisms have been the focus of depression research, whereas the VTA to accumbens dopaminergic pathway is a focus in addiction research. This is because virtually all drugs of abuse increase dopamine neurotransmission in the nucleus accumbens, partially mediating their rewarding effects (Koob *et al.*, 1998; Wise, 1998). However, the distinctions between depression and addiction research are artificial, and there is a need to systematically examine the role of VTA-accumbens in depression. One approach has been the use of animal models. For example, decreased cytochrome oxidase activity in the VTA and nucleus accumbens of congenitally helpless rats suggests impaired reward processing in depression (Shumake *et al.*, 2003).

1.9.1.9 Dorsal raphe

The midbrain raphe nuclei are the principal source of cerebral serotonin (Harding *et al.*, 2004) and serotonergic neurons from the dorsal raphe project to the upper brain stem, hypothalamus, thalamus, and cerebral cortex (Kandel, 2000). The serotonergic hypothesis of mood disorders is supported by experiments in which transient depressive relapses are induced by a reduction in the level of plasma tryptophan, the amino acid precursor of serotonin (5-HT) (Delgado *et al.*, 1990; Smith *et al.*, 1997). Even though there is strong evidence that a disturbance of serotonergic function underlies human mood disorders, the involvement of the raphe nuclei in the pathophysiology of depression remains unclear (Coppen, 1967; Lowry *et al.*, 2008; Meltzer, 1990). Interestingly, activation of serotonin receptors and 5-HT release from the dorsal raphe appear necessary for development of learned helplessness in the rat (Grahn *et al.*, 1999; Grahn *et al.*, 2000; Maier & Watkins, 2005; Petty *et al.*, 1994).

1.9.1.10 Habenula and interpeduncular nucleus

Human neuroimaging studies of depression report increased thalamic metabolism (Drevets *et al.*, 1992; Morris *et al.*, 1999; Saxena *et al.*, 2001). Morris et al. (1999) further localized the source to a region immediately superior and lateral to the posterior commissure, which most likely corresponds to the habenula nucleus (Mai *et al.*, 2008).

The habenula, an epithalamic structure, showed hypermetabolism in several animal models of depression (Caldecott-Hazard *et al.*, 1988) including the congenitally helpless rat (Shumake *et al.*, 2003). Furthermore, two human neuroimaging studies have shown increased habenula metabolism or blood flow correlating with depressive symptoms after acute tryptophan depletion (Morris *et al.*, 1999; Rosier *et al.*, 2009). In

addition, habenula ablation completely blocked the development of learned helplessness in rats (Amat *et al.*, 2001). The habenula, which is generally divided into lateral and medial aspects in the rat (Paxinos & Watson, 1996), has been largely overlooked in depression research. However, due to its major influence on monoamine transmission (Hikosaka *et al.*, 2008; Wang & Aghajanian, 1977) it may play an important role in depression. Furthermore, animal studies have implicated the habenula in variety of processes disrupted in depression including sleep-wake cycle (Haun *et al.*, 1992; Valjakka *et al.*, 1998), reward mechanisms (Boyd & Celso, 1970; Gallistel *et al.*, 1985; Sutherland & Nakajima, 1981), antinociception (Benabid & Jeaugey, 1989; Fuchs & Cox, 1993), behavioral inhibition (Lee & Huang, 1988) and hormonal responses to stress (Sutherland, 1982; Sandyk, 1991).

The interpeduncular nucleus (IPN) which receives a major projection from the medial habenula (Contestabile & Flumerfelt, 1981) was metabolically hyperactive in congenitally helpless rats and may contribute to helplessness susceptibility (Shumake *et al.*, 2003). The IPN receives more acetylcholine input than any other region in the mammalian brain (Woolf & Butcher, 1985) and there is evidence that excessive cholinergic activity is implicated in the etiology of depression (Charles *et al.*, 1994; Dilsaver & Coffman, 1989; Janowsky *et al.*, 1983; Steingard *et al.*, 2000).

The neural correlates of helpless behavior including analysis of regional functional connectivity differences between helpless and non-helpless Holtzman rats were identified and reported in Chapter 5. Metabolic activity was examined using cytochrome oxidase histochemistry.

1.10 OVERVIEW

The long-term goal is to determine the neurobiological mechanisms that underlie stress vulnerability and response to antidepressant treatment. The objectives of this

dissertation were to 1) identify a stress-susceptible rat strain and characterize the behavioral predictors of helplessness susceptibility by studying three dimensions of temperament (reward dependence, novelty-seeking and harm avoidance) before stress exposure, 2) identify the neural network effects of response and non-response to chronic fluoxetine treatment using a stress-susceptible animal model, and 3) determine the neurophysiologic correlates of helplessness susceptibility. This was examined via measurement of regional brain metabolic capacity and functional connectivity within relevant neural circuits. Responsivity of the HPA axis and autonomic nervous system to stress were also assessed. These effects were studied in a nonselectively-bred rat strain that underwent inescapable shock exposure followed by escape testing. The overarching hypothesis is that animals with a genetic predisposition to stress-induced helplessness will show behavioral and neurophysiologic patterns that are predictive of helpless behavior. This dissertation work is relevant because predisposed animals revealed patterns in neural metabolic activity that correlated with antidepressant treatment response. In general, diagnosis and treatment of disorders such as diabetes and hypertension follow standardized evidence-based guidelines that rely on the collection of data from physiologic markers in combination with known family history of disease. However, a similar approach is not commonly used in the diagnosis and treatment of several psychopathologies including depression and anxiety. Identification of bio-behavioral factors that correlate with helplessness such as brain activity patterns, novelty-seeking and heart rate measurements could be incorporated into clinical guidelines together with genetic or hereditary information to help empirically determine diagnosis and treatment plans of individuals that might have increased vulnerability to stress-related disorders.

Animal models for human diseases are generally evaluated by meeting three criteria: face validity, predictive validity, and construct validity (Willner, 1984; Willner & Mitchell, 2002). Face validity means that the phenotype of the animal model should be highly similar to the human disease characteristics. Predictive validity describes whether drugs that have shown specificity and efficacy in humans will similarly affect the disease phenotype in the model. Construct validity is probably the hardest criterion to fulfill as the construct of disease is often still unclear and is the object of investigation. However, if specific risk factors have been identified for a disease, they should be evaluated in a potential animal model. The order of the chapters will be presented as follows: Chapter 2 addresses *face validity* and presents data on the genetic predisposition to helpless behavior between three strains of outbred rats: Holtzman, Sprague Dawley and Long-Evans. Chapter 3 addresses *predictive validity* and describes the limbic-cortical network differences between responders and non-responders to fluoxetine antidepressant treatment among stress-susceptible rats. Chapters 4 and 5 address *construct validity.* Chapter 4 presents the behavioral and physiological correlates of stress susceptibility and resistance among the Holtzman strain and Chapter 5 presents an analysis of the neural mechanisms underlying susceptibility to helplessness in terms of regional metabolic activity and functional connectivity. Finally, Chapter 6 offers a summary and integration of the major findings, a brief discussion of their implications, and potential applications to current clinical practice.

Chapter 2 Strain, Sex, and Open-Field Behavior: Factors Underlying the Genetic Susceptibility to Helplessness

Learned helplessness represents a failure to escape after exposure to inescapable stress and may model human psychiatric disorders related to stress. Previous work has demonstrated individual differences in susceptibility to learned helplessness. In this study, we assessed different factors associated with this susceptibility, including strain, sex, and open-field behavior. Testing of three rat strains (Holtzman, Long-Evans, and Sprague Dawley) revealed that Holtzman rats were the most susceptible to helplessness. Holtzman rats not only had the longest escape latencies following inescapable shock, but also showed spontaneous escape deficits in the absence of prior shock when tested with a fixed-ratio 2 (FR2) running response. Moreover, when tested with fixed-ratio 1 (FR1) running—an easy response normally unaffected by helplessness training in rats—inescapable shock significantly increased the escape latencies of Holtzman rats. Within the Holtzman strain, we confirmed recent findings that females showed superior escape performance and therefore appeared more resistant to helplessness than males. However, regression and covariance analyses suggest that this sex difference may be explained by more baseline ambulatory activity among females. In addition, some indices of novelty reactivity (greater exploration of novel vs. familiar open-field) predicted subsequent helpless behavior. In conclusion, Holtzman rats, and especially male Holtzman rats, have a strong predisposition to become immobile when stressed which interferes with their ability to learn active escape responses. The Holtzman strain therefore appears to be a commercially available model for studying susceptibility to helplessness in males, and novelty-seeking may be a marker of this susceptibility.

2.1 INTRODUCTION

Learned helplessness (LH) represents a failure to exhibit an escape response after exposure to inescapable stress (Overmier & Seligman, 1967). This paradigm serves as a useful tool to model stress-induced psychopathology, such as depression or post-traumatic stress disorder (PTSD) (Foa *et al.*, 1992; Petty *et al.*, 1996; Petty *et al.*, 1997; Seligman, 1975b). However, there are individual differences in the response to stress because most individuals do not develop psychopathology in response to psychological stress or trauma (Breslau, 2001; Kendler *et al.*, 1999; Kessler *et al.*, 1995; Nestler *et al.*, 2002). Therefore, there is a need to identify biological factors which confer vulnerability to stress-induced psychopathology. Identifying these factors in humans is difficult because most human studies have examined individuals only after stress has taken its toll. Animal models provide a convenient way to investigate the predisposing factors underlying susceptibility to helplessness. One successful strategy has been to study rat lines selectively bred for behaviors which model human psychiatric disorders (Henn & Edwards, 1994; Shumake *et al.*, 2005). For example, rats selectively bred to display LH show neurological and behavioral signs similar to those seen in humans with depression and PTSD (Shumake *et al.*, 2000; Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). However, selective breeding protocols can take years to develop and can be burdensome to maintain. Therefore, it would be beneficial if there were a commercially-available strain with increased susceptibility to LH. While there are a few reports of increased anxious or depressed behavior in some inbred rat strains such as the Wistar-Kyoto (Braw *et al.*, 2006), to our knowledge only one study has assessed different susceptibilities to LH among different outbred strains. Wieland et al. (1986) found that Holtzman rats were twice as likely to develop learned helplessness as Sprague Dawley rats. Our first objective was to see if this susceptibility difference between strains

reported over 20 years ago was still present today. In addition, we assessed the susceptibility of Long-Evans rats, which, to our knowledge, have never been evaluated in the learned helplessness paradigm.

As our results will show, we verified that the Holtzman line still shows the most helpless behavior following inescapable shock. Our next objective was to evaluate whether the escape deficits exhibited by Holtzman rats were in fact induced by prior exposure to inescapable shock, or whether they reflect a baseline deficit in escape learning (i.e., learned vs. spontaneous helplessness). For example, after multiple generations of selective breeding for learned helpless behavior, the majority of rats show spontaneous escape deficits regardless of whether they are exposed to inescapable shock beforehand (Henn & Vollmayr, 2005). Rats from this selected line also show a number of other behavioral differences, such as hyperactivity specific to novel environments (Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). We have interpreted these behaviors as reflecting a temperament of high novelty-seeking, which in humans has been associated with risk of developing PTSD (Richman & Frueh, 1997; Wang *et al.*, 1997). However, to our knowledge, only one study has examined whether individual differences in temperament can predict the development of helplessness in rats, and this study came to essentially the opposite conclusion that reluctance to explore a novel open field predicted vulnerability to helplessness (Minor *et al.*, 1994). Therefore, our third objective was to use linear regression to evaluate whether open-field activity before inescapable shock could predict escape deficits after inescapable shock.

Our final objective was to assess sex as a risk factor for helplessness. Despite consistent reports of increased incidence of PTSD and depression in women (Breslau, 2001; Weissman *et al.*, 1993), there are conflicting reports of sex differences in the development of learned helplessness in rats. One study found that female rats

expressed more helpless behavior than males depending on estrus phase (Jenkins *et al.*, 2001), while another study found less helpless behavior in females, independent of gonadal hormones (Dalla *et al.*, 2008). To address this issue, we evaluated both males and females with measures of temperament and helplessness and assessed whether the phase of estrus cycle contributed to the expression of helpless behavior.

2.2 MATERIALS AND METHODS

2.2.1 Subjects

Subjects were 41 male Holtzman, 10 male Sprague Dawley, 10 male Long-Evans and 41 female Holtzman rats obtained from Harlan (Madison, WI) at postnatal day 30 (P30). Animals were housed 2-3 per cage and maintained on a 12h/12h light/dark photoperiod in a facility accredited by the Association for the Assessment of Laboratory Animal Care International. Food and water were available *ad libitum*. Subjects were handled and weighed for five minutes every day for one week prior to starting behavioral experiments. Open-field experiments occurred between 0900 h and 1200 h. Inescapable shock training occurred between 0900 h and 1700 h and escapable shock testing was performed between 0800 h and 1900 h. Experiments were done in accordance with NIH guidelines for the use of experimental animals and were approved by the University of Texas Institutional Animal Care and Use Committee.

2.2.2 Apparatus

The open-field chamber (43.2 x 43.2 cm) consisted of clear plastic sides 30.5 cm high and a white plexiglass floor. Activity was detected by arrays of infrared light beam motion detectors (16 x 16, 2.5 cm apart) at the sides of each chamber, thus creating a detection grid. Two arrays of detectors were located 1 cm above the floor, and another array was located 13 cm above the floor, to detect rearing. The chambers were

controlled by the Activity Monitor program, version 5.10 (Med Associates, St. Albans, VT).

Two inescapable shock chambers (30 x 25 x 20 cm) (Med Associates, St. Albans, VT) were enclosed in sound-attenuated boxes and illuminated by a red light. Each apparatus had two sides of aluminum, with clear plexiglass for the front, back, and top. A soapy solution made from Ivory dishwashing liquid (Procter and Gamble, Cincinnati, OH) was placed in the tray beneath the chambers to provide a distinct olfactory cue for the inescapable context. Shocks were delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). The chamber was controlled by MED-PC, version 4 (Med Associates, St. Albans, VT), using a program written in the MEDSTATE language.

The shuttle box (42 x 16 x 25 cm) consisted of two compartments of equal size, separated by a door (11 x 9 cm) that remained open throughout the session. The chamber was enclosed in a sound-attenuated box and illuminated by a white light (10 lux). Two sides of the chamber were aluminum, with clear plexiglass for the front, back, and top. Shocks were delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). The subject's position was detected by eight sets of infrared light beam motion detectors, located 2 cm above the grid floor, spaced 4.4 cm apart from each other, on both sides of the chamber. The chamber was controlled by MED-PC, version 4, using a program written in the MEDSTATE language. This program used beam breaks of the two pairs of beams located at either end of both sides of the chamber as the contingency for terminating shock, to score a complete crossing. A povidone-iodine solution (First Priority, INC., Elgin, IL) was placed in the tray beneath the chamber to provide a distinct olfactory cue for the escapable context.

2.2.3 Behavioral experiments

Ten separate cohorts with a total number of 102 subjects underwent behavioral testing for 11 weeks. Within each cohort behavioral experiments took place during four consecutive days. All animals were tested for open-field (OF) activity during the first day of experiments (novel OF) on P40 to determine if behavioral characteristics predictive of helplessness were present before learned helplessness training. Each animal was placed in the same corner of the open-field chamber and behavior was recorded for 10 minutes. The chambers were washed with a diluted Bio-clean detergent solution (Stanbio laboratory, Boerne, TX) between each session. Measures included ambulatory counts (horizontal beam breaks), average velocity, rearing counts (vertical beam breaks), average rearing duration, and thigmotaxis (time spent in the 62% periphery versus the 38% center of the open-field). Measures were automatically scored by a computer using MED-PC software.

On the second day, subjects were re-tested in the open-field (familiar OF). From these two open-field sessions, behavioral measures were calculated reflecting both general activity (novel plus familiar) and novelty-specific activity (novel-to-familiar ratio). Thus, an animal with high exploratory activity in both the novel and familiar open fields would have a high general-activity score but a low novelty-reactivity score. In order to have a high novelty-reactivity score, an animal would have to be very active in the novel open-field but much less active in the familiar open-field. Thus, these two scores help to separate animals which are excited by novel environments from those that are chronically hyperactive.

On the third day, eighty-two subjects (31 male Holtzman, 10 male Long-Evans, 10 male Sprague Dawley and 31 female Holtzman), were trained in the inescapable shock chamber to observe the helpless phenotype (Hunziker & Dos Santos, 2007). Each

session included 60 trials of 10 s duration with pseudorandom inter-trial intervals ranging from 10 to 110 seconds. To find the minimum shock intensity that would induce helplessness and motivate escape behavior, we tried three different shock intensities: 0.5 (n=11), 0.75 (n=40), and 1.0 mA (n=11). An ANOVA revealed no significant effect of amperage [$F(2,59) = 0.521$, $p = 0.60$] on escape performance, and the mean FR2 escape latencies for 0.5 mA (17 s), 0.75 mA (20 s), and 1.0 mA (18 s) were virtually equivalent. Therefore, subjects were pooled together for the statistical analyses without regard to amperage. In addition, in order to test the effects of prior shock exposure on escape behavior, 10 male and 10 female Holtzman rats did not receive inescapable shock prior to helplessness testing in the shuttle box.

On the fourth day, subjects were tested with the escapable shock paradigm using a shuttle box to measure escape behavior. First, subjects were tested with five trials of a fixed-ratio (FR) 1 schedule consisting of crossing from one side of the box to the other to terminate the shock. This was followed by 25 trials of an FR2 schedule in which animals had to cross twice; in other words, rats had to return to the compartment where the shock was initiated in order to terminate the shock. The maximum possible footshock duration was 30 seconds, after which the shock was terminated if the subject had not escaped. Pseudorandom inter-trial intervals consisted of durations ranging from 10 to 110 seconds. Amperage was matched to what was delivered in the inescapable session, as discussed above. Number of escape failures, latency to perform the first cross during FR2, and escape latency (time to terminate the footshock) were automatically scored using MED-PC software.

2.2.4 Procedure for determining phase of estrus cycle

Daily vaginal smears were taken on the days of open field tests, inescapable shock training and escapable shock testing. Tapered-end cotton swabs were immersed

in a beaker filled with 10 ml of tap water. Using the moistened swab, samples were taken from the vaginal wall and smeared over gelatin-coated slides. Samples were allowed to dry for 24 hours and vaginal epithelial cells were imaged using an Olympus light microscope at 10x magnification and 0.25 NA. The imaging process was used to classify the reproductive phase of the female rats into estrus, diestrus or proestrus (Nelson R.J., 2000).

2.2.5 Statistical analysis

Strain differences in open-field activity were analyzed using both sums (novel plus familiar) and ratios (novel to familiar) of the two open-field sessions. The former served as an index of general activity, while the latter reflected the proportion of activity that was novelty specific. Analysis of variance (ANOVA) was used to test for an overall effect of strain in these parameters, and Tukey's post-hoc test was used to test for differences between individual strains. Helplessness testing was analyzed with repeated measures ANOVA, using the FR1 and FR2 escape latencies as two separate levels in a within-subject variable. In addition to the final (second cross) escape latencies of the FR2 trials, latencies to make the first crossing were also analyzed with ANOVA, with Tukey's post-hoc test as a follow-up. An analysis of covariance (ANCOVA) tested whether controlling for ambulation in the open-field would have an effect on the escape latency results. Effects of prior shock exposure and sex differences were analyzed with repeated measures ANOVA as described above. ANOVAs were used to analyze sex differences in open-field activity and differences in escape latency related to estrus cycle. A series of linear regressions were calculated between average escape latency and open-field parameters, to determine if the prior behavior of each subject would be predictive of helpless behavior.

2.3 RESULTS

2.3.1 Strain differences

2.3.1.1 Strain differences in open-field behavior

The three strains were tested in an open-field apparatus across two consecutive days (novel and familiar sessions) prior to exposure to any stressor. The locomotor activity was scored in terms of ambulation, rearing, and time spent in the center of the field. Then two types of scores were computed: a general-activity score, calculated by summing the activity scores from both novel and familiar sessions, and a novelty-reactivity score, calculated by dividing activity measures in the novel session by the same measures in the familiar session. The means and standard errors for general activity and the novelty-specific index are presented in the top and bottom halves, respectively, of Table 2.1. Only one general activity parameter was significant: average rearing duration, $F(2,26) = 7.19$ ($p < 0.01$), which Tukey's post-hoc test showed to be significantly greater in the Long-Evans subjects as compared to the Sprague Dawley subjects ($p < 0.01$).

Table 2.1 Mean open-field activity by rat strain

	Holtzman (n=9)	Long-Evans (n=10)	Sprague-Dawley (n=10)
Open Field Activity Parameter	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
GENERAL ACTIVITY (Sum of Novel and Familiar Sessions)			
Total Ambulatory Counts	1835 ± 239	1907 ± 291	2649 ± 245
Average Velocity (cm/sec)	2.08 ± 0.16	2.11 ± 0.23	2.21 ± 0.12
Total Rearing Counts	222 ± 34.8	224 ± 29.1	309 ± 28.1
Average Rearing Duration (sec)	1.14 ± 0.08	1.36 ± 0.13 *	0.84 ± 0.06
Center Zone Time (sec)	129 ± 20.3	111 ± 21.3	104 ± 15.2
NOVELTY-SPECIFIC INDEX (Ratio of Novel : Familiar Sessions)			
Total Ambulatory Counts	0.93 ± 0.08	1.13 ± 0.22	1.43 ± 0.20
Average Velocity (cm/sec)	0.99 ± 0.13	0.99 ± 0.12	1.27 ± 0.26
Total Rearing Counts	0.98 ± 0.08	0.98 ± 0.17	1.63 ± 0.35
Average Rearing Duration (sec)	0.92 ± 0.16	0.96 ± 0.09	0.98 ± 0.22
Center Zone Time (sec)	0.65 ± 0.12	1.73 ± 0.53	2.05 ± 0.73

* LE greater than SD, $p < 0.05$

2.3.1.2 Strain differences in escape latency

Repeated measures ANOVA (3 x 2), with strain (Holtzman, Sprague Dawley, and Long-Evans) as a between-subject variable and response contingency (average of FR1 vs. average of FR2 trials) as a within-subject variable, tested whether strain differences would give rise to differences in escape behavior. There was a significant interaction between strain and response contingency, $F(2,26) = 19.556$ ($p < 0.001$), indicating that Sprague Dawley subjects maintained low escape latencies across both FR1 and FR2 trials while the other two strains showed an increase in escape latencies during the FR2 trials (Fig. 2.1). There was also a significant main effect of strain, $F(2,26) = 9.12$ ($p < 0.01$), with the Holtzman (18 ± 2 s) and Long-Evans (13 ± 2 s) strains showing significantly greater escape latencies than the Sprague Dawley (4 ± 1 s) strain ($p < 0.01$). However, there was no significant difference between the Holtzman and Long-Evans strains.

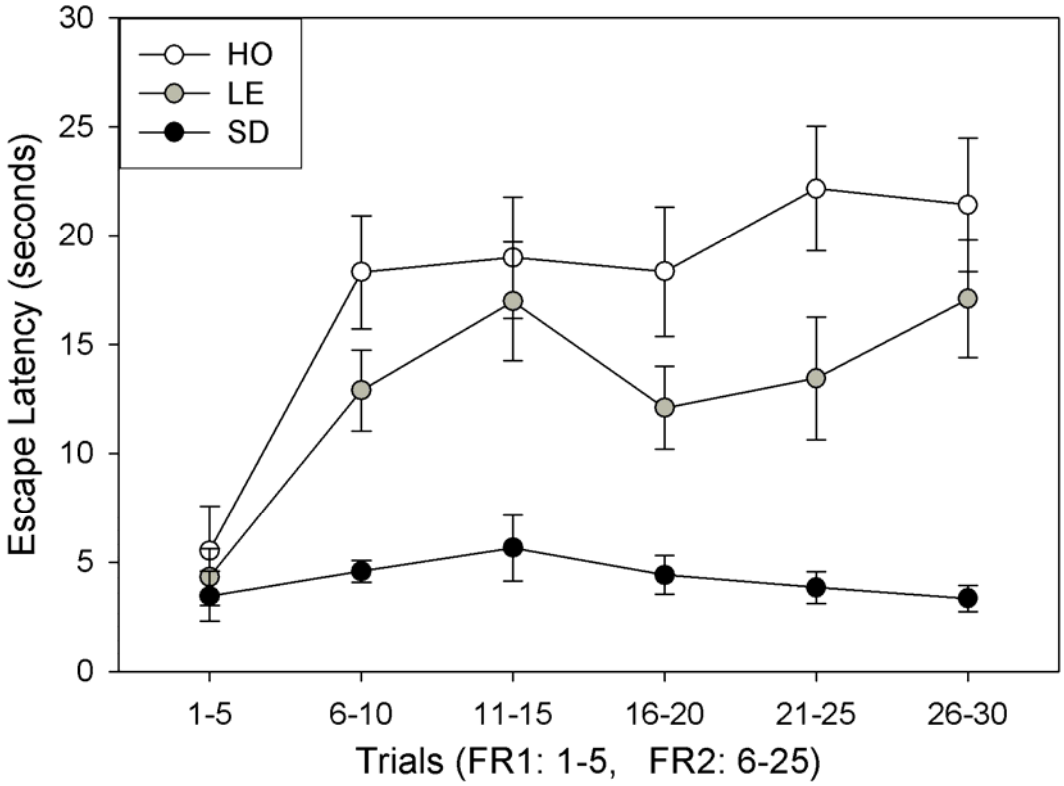

Figure 2.1 Mean ± standard error escape latencies (in seconds) during helplessness testing of Holtzman (HO, n = 9), Long-Evans (LE, n = 10), and Sprague Dawley (SD, n = 10) subjects. The first five trials represent a fixed-ratio-one (FR1) contingency, and the last 25 trials represent a fixed-ratio-two (FR2) contingency.

The average latency to perform the first crossing of the fixed-ratio-two (FR2) response also showed a significant group difference, $F(2,26) = 12.4$ ($p < 0.001$). The Holtzman (14 ± 3 s) subjects took longer to make this first crossing than did either Long-Evans (7 ± 1 s) or Sprague Dawley (2 ± 0.3 s) subjects ($p < 0.05$), but the Long-Evans and Sprague Dawley strains were not significantly different from each other. Another ANOVA showed a significant group difference in the number of failures to terminate the shock in less than 30 seconds, $F(2,26) = 6.78$ ($p < 0.01$). The Holtzman (34% failed trials) and Long-Evans (27% failed trials) rats had significantly more failures than Sprague Dawley (2% failed trials) subjects ($p < 0.05$), but were not significantly different from each other.

2.3.2 Effect of prior shock exposure in Holtzman rats

The next analysis assessed the effects of prior shock exposure for both sexes, testing whether twenty Holtzman subjects which did not undergo inescapable shock ("naïve group") showed escape latencies that were significantly different from the sixty-two Holtzman rats which did undergo inescapable shock ("prior shock group"). A repeated measures ANOVA (2 x 2 x 2) used group and sex as between-subject variables, and response contingency (FR1 vs. FR2) as a within-subject variable. A significant interaction between group and response contingency was found, $F(1,78) = 11.0$, $p < 0.01$. Simple-effects tests showed that the FR1 contingency was sensitive to the effect of prior shock exposure while the FR2 contingency was not (Fig. 2.2).

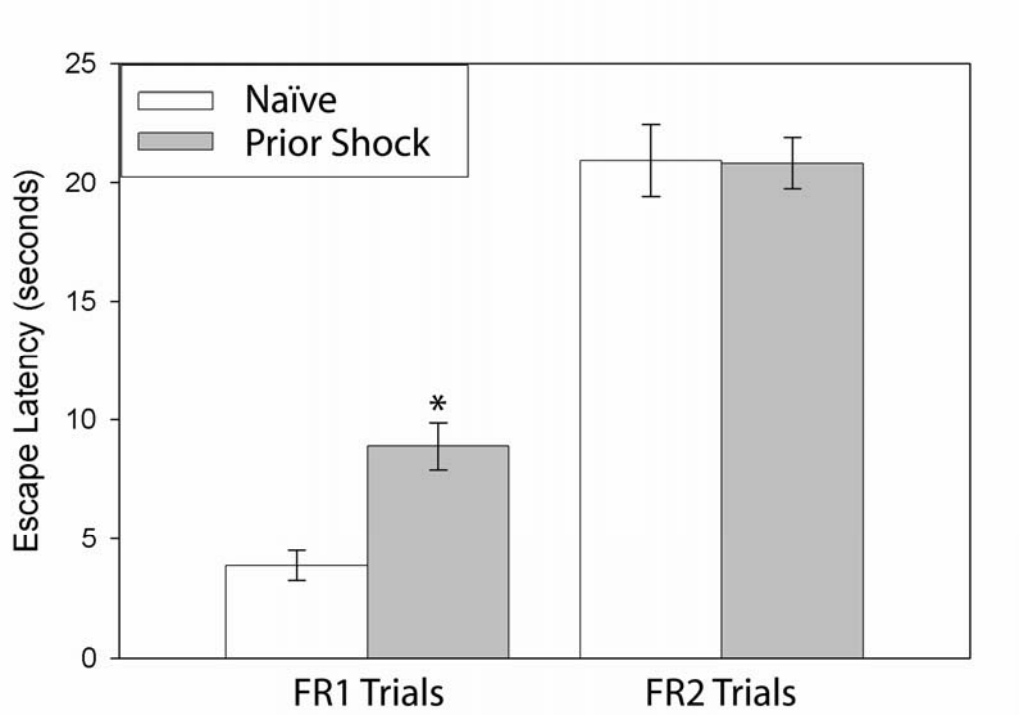

Figure 2.2 Mean ± standard error escape latencies (in seconds) during the 5 FR1 trials and 25 FR2 trials, for Holtzman subjects assigned to either the naïve group (n = 20), which did not receive inescapable footshock exposure prior to helplessness testing in the shuttle box, or the prior shock group (n = 62), which did receive inescapable shock prior to testing. * $p < 0.01$.

2.3.3 Sex differences

2.3.3.1 Sex differences in escape latency

The repeated measures ANOVA (2 x 2 x 2), which was used to analyze prior shock exposure above, found no interaction between sex and inescapable shock, but it did find a significant interaction between sex and response contingency, $F(1,78) = 7.32$, $p < 0.01$. Simple-effects tests of the interaction showed sex differences with the FR2 contingency but not with the FR1 contingency, with females performing better than males in the more difficult FR2 task (Fig. 2.3). Average latency to make the first crossing of the FR2 response, as well as average failure rate, showed similar trends in sex differences, but neither effect was significant, Fs(1,60) = 2.14 and 1.85, respectively.

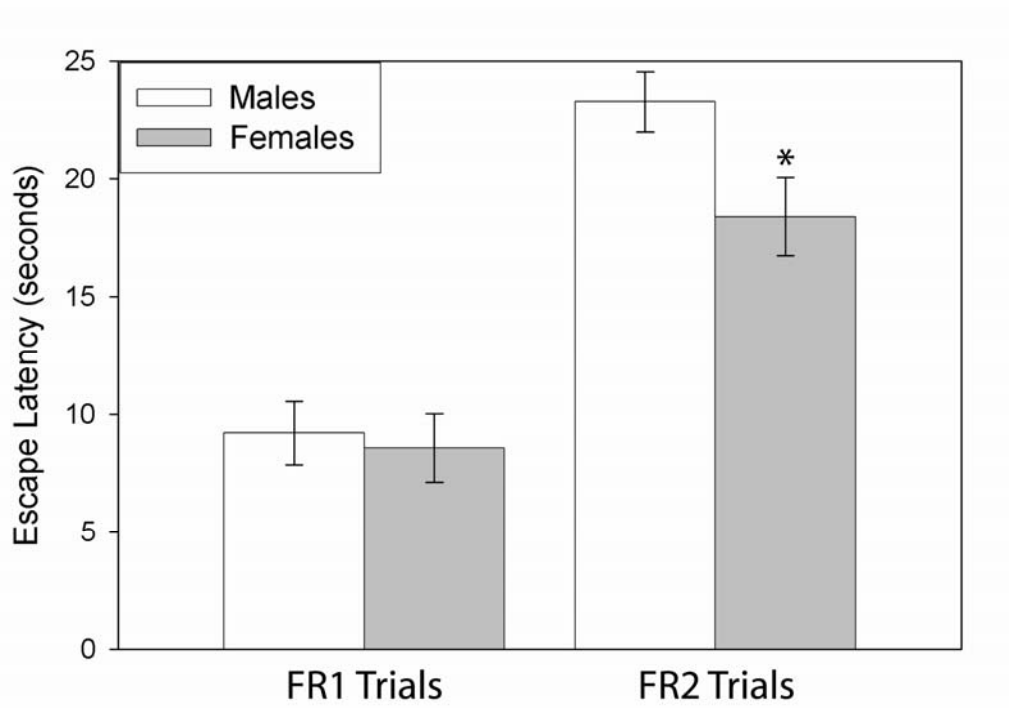

Figure 2.3 Mean ± standard error escape latencies (in seconds) during the 5 FR1 trials and 25 FR2 trials, for both male (n = 31) and female (n = 31) Holtzman subjects, all of which received inescapable footshock prior to helplessness testing in the shuttle box. * $p < 0.05$.

2.3.3.2 Estrus cycle and escape latency

To test whether a subject's estrus cycle had an effect on the development of helplessness, the 31 female subjects in the prior shock group were given vaginal smears on both the day of inescapable shock presentation and the subsequent day of shuttle box helplessness testing. On the day of inescapable shock training, there were 13 rats in estrus, 11 rats in diestrus, and 7 rats in proestrus. On the day of escapable shock testing, there were 15 rats in estrus, 11 rats in diestrus, and 5 rats in proestrus. One-way ANOVAs tested whether the estrus phase had a significant effect on escape latencies. The estrus phase during inescapable shock had no significant effect on either FR1 or FR2 escape latencies, *F*s(2,28) = 0.150 and 0.103, respectively. The estrus phase during helplessness testing also had no effect on either FR1 or FR2 latencies, *F*s (2,28) = 1.01 and 0.488, respectively. Average first crossing latency and average failure rate were similarly unaffected by estrus cycle.

2.3.3.3 Sex differences in open-field activity

A series of ANOVAs analyzed the open-field activity of the Holtzman subjects (n = 41 males, 41 females) for sex differences in the same locomotor parameters used for the analysis of strain differences. The means and standard errors for these parameters for male and female Holtzman rats are shown in Table 2.2. The ANOVAs on the novel-plus-familiar index of general activity found that the females reared more than males, $F(1,80) = 14.5$, $p < 0.01$. The ANOVAs on novelty-specific activity (novel-to-familiar ratio) found no significant sex differences.

Table 2.2 Mean open-field activity of male and female Holtzman rats

Open Field Activity Parameter	Males (n = 41) Mean ± S.E.	Females (n = 41) Mean ± S.E.
GENERAL ACTIVITY (Sum of Novel and Familiar Sessions)		
Total Ambulatory Counts	1893 ± 100	2210 ± 128
Average Velocity (cm/sec)	2.06 ± 0.07	2.19 ± 0.07
Total Rearing Counts	232 ± 14.1	320 ± 18.2 **
Average Rearing Duration (sec)	1.01 ± 0.04	1.04 ± 0.05
Center Zone Time (sec)	126 ± 9.8	125 ± 8.6
NOVELTY-SPECIFIC INDEX (Ratio of Novel : Familiar Sessions)		
Total Ambulatory Counts	1.26 ± 0.09	1.47 ± 0.09
Average Velocity (cm/sec)	1.08 ± 0.06	1.25 ± 0.10
Total Rearing Counts	1.28 ± 0.08	1.47 ± 0.13
Average Rearing Duration (sec)	0.95 ± 0.06	0.98 ± 0.06
Center Zone Time (sec)	1.50 ± 0.22	1.72 ± 0.29

** $p < 0.01$

2.3.4 Prior behavior predicting LH response

2.3.4.1 Individual differences within the Holtzman strain

Previous work investigating individual differences in stress vulnerability has consistently demonstrated bimodal distributions, with most subjects either very resistant or very vulnerable and few subjects in between (Drugan *et al.*, 1989; Levay *et al.*, 2006). We calculated a frequency distribution using the percentage of failed trials for the 82 Holtzman subjects which underwent helplessness testing (Fig. 2.4). As predicted, two peaks are seen, with most subjects performing either very well or very poorly on the escape task.

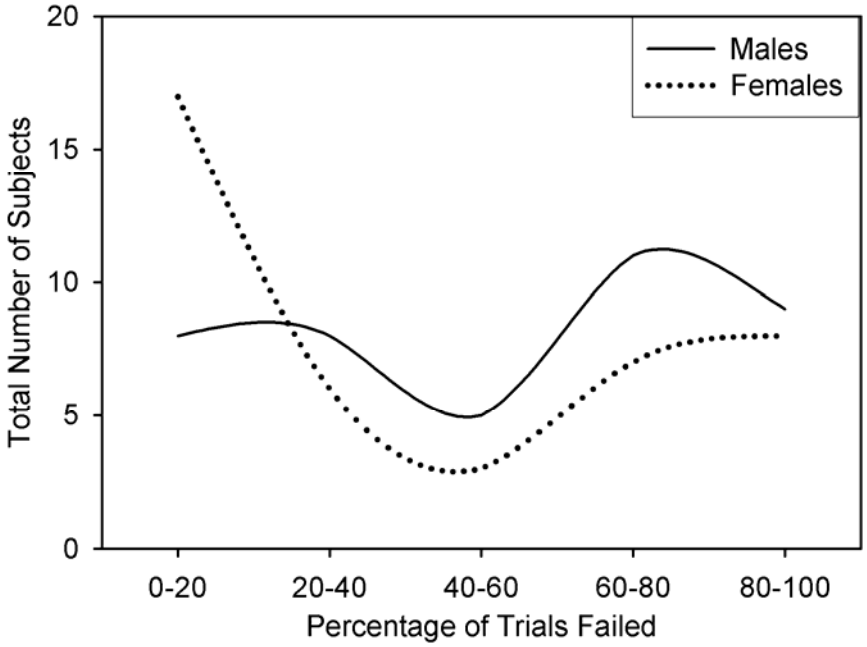

Figure 2.4 Frequency distributions of the total number of Holtzman subjects that failed a given percentage of FR2 trials in the shuttle box. Subjects were grouped into bins of 20% increments, from 0% to 100% failure rate. The black line indicates number of male subjects (n = 41); the dotted line indicates number of female subjects (n = 41).

2.3.4.2 Regression with open-field behavior

To determine if any locomotive behaviors during open-field testing were predictive of the phenotype eventually exhibited by these subjects, a series of linear regressions was computed with each locomotive parameter as the independent variable and the FR1 and FR2 average escape latencies as the dependent variables (Table 2.3). In general, the regression analyses indicated that subjects with higher general activity had shorter escape latencies when tested in the escape task. Multiple linear regression analyses indicated that ambulation, velocity, and rearing collectively explained 11% of the variance in FR1 escape latencies and 18% of the variance in FR2 escape latencies for animals which received inescapable shock. However, the opposite relationship was observed for novelty-specific measures, with higher novelty-specific scores predicting longer FR1 escape latencies. Specifically, ambulation, rearing, and center zone time collectively explained 12% of the variance in FR1 escape latencies. The fact that this relationship is observed only in subjects which received inescapable shock (prior shock but not naïve) and only with the response schedule which showed an effect of inescapable shock (FR1 but not FR2) suggests that the novelty-specific index is associated with helplessness vulnerability and not merely the baseline ability to perform the task.

Table 2.3 Standardized beta (β) coefficients for open-field variables predicting escape latencies in prior shock versus naïve Holtzman subjects.

	Prior Shock (n = 62)		Naïve (n = 20)	
Open Field Activity Parameter	FR 1 Trials	FR 2 Trials	FR 1 Trials	FR 2 Trials
GENERAL ACTIVITY (Sum of Novel and Familiar Sessions)				
Total Ambulatory Counts	-0.30 *	-0.40 **	-0.12	-0.48 *
Average Velocity (cm/sec)	-0.30 *	-0.34 **	-0.07	-0.23
Total Rearing Counts	-0.27 *	-0.37 **	-0.23	-0.45 *
Average Rearing Duration (sec)	-0.20	-0.12	-0.25	-0.40
Center Zone Time (sec)	-0.19	-0.15	-0.18	-0.27
NOVELTY-SPECIFIC INDEX (Ratio of Novel : Familiar Sessions)				
Total Ambulatory Counts	0.31 *	0.07	0.02	-0.06
Average Velocity (cm/sec)	0.23	0.11	0.33	0.26
Total Rearing Counts	0.30 *	0.18	0.03	0.04
Average Rearing Duration (sec)	0.07	0.11	0.17	0.18
Center Zone Time (sec)	0.33 **	0.16	-0.12	0.06

** $p < 0.01$; * $p < 0.05$

2.3.4.3 Sex differences in regression with open-field activity

To take into account the possibility that sex could mediate the relationship between open-field and escape behavior, the above regression analyses were repeated separately for males and females (for only Holtzman subjects receiving inescapable shock). As before, beta coefficients for both general activity and novelty-specific activity were calculated (Table 2.4). Although the specific measures reaching statistical significance vary by sex and response contingency, the overall pattern of results was unchanged, with general activity predicting better escape performance and novelty-specific activity predicting worse escape performance for both males and females. However, eliminating the variance associated with sex improves the predictability of male behavior. For example, a multivariate function which combines measures of ambulation, velocity, rearing, and center-zone exploration shows that general activity accounts for 28% of the variance in the FR1 escape latencies of males, but only 12% for that of females. Novelty-specific activity is an even better predictor of FR1 activity, accounting for 33% of the variance of males and 20% of the variance of females.

Table 2.4 Standardized beta (β) coefficients for open-field variables predicting escape latencies in male versus female Holtzman subjects.

	Males (n = 31)		Females (n = 31)	
Open Field Activity Parameter	FR 1 Trials	FR 2 Trials	FR 1 Trials	FR 2 Trials
GENERAL ACTIVITY (Sum of Novel and Familiar Sessions)				
Total Ambulatory Counts	-0.26	-0.27	-0.32	-0.47 **
Average Velocity (cm/sec)	-0.42 *	-0.34	-0.20	-0.36 *
Total Rearing Counts	-0.24	-0.11	-0.29	-0.41 *
Average Rearing Duration (sec)	-0.25	-0.07	-0.18	-0.23
Center Zone Time (sec)	-0.11	-0.04	-0.30	-0.36 *
NOVELTY-SPECIFIC INDEX (Ratio of Novel : Familiar Sessions)				
Total Ambulatory Counts	0.22	0.15	0.43 *	0.21
Average Velocity (cm/sec)	0.01	0.04	0.36 *	0.26
Total Rearing Counts	0.36 *	0.39 *	0.32	0.23
Average Rearing Duration (sec)	0.23	0.29	-0.03	0.11
Center Zone Time (sec)	0.47 **	0.24	0.34	0.29

** $p < 0.01$; * $p < 0.05$

2.3.5 Analysis of covariance

Since the regression analyses suggested that baseline differences in activity level can explain some of the variance in escape latency, we were interested to see if the mean differences in escape latency attributed to strain and sex could be due to baseline activity differences. That is, there was a general trend for reduced activity in the Holtzman strain and increased activity in females, which might explain the greater and lesser vulnerability to helplessness exhibited by Holtzman rats and females, respectively. In order to address this possibility, analyses of covariance (ANCOVAs) were performed on the FR2 trials, using total ambulatory counts as a covariate, to see if controlling for individual differences in open-field ambulation would eliminate strain and sex differences in escape latency. There was still a highly significant effect of strain on escape latency, $F(2,25) = 15.0$, $p < 0.001$, with Sprague Dawley rats performing better than either Long-Evans or Holtzman strains. However, the effect of sex on escape latency was no longer significant, $F(1,77) = 1.16$. Therefore, the observed strain differences in escape behavior cannot be attributed to strain differences in activity level, but the apparent resilience of females in the escape test may be attributed to their hyperactivity relative to males.

2.4 DISCUSSION

2.4.1 Holtzman rats appear most predisposed to helplessness

We were able to replicate one of Wieland et al. (1986) main findings: namely, Holtzman rats showed greater escape latencies compared to Sprague Dawley rats. In addition, we found that Long-Evans rats, which had been previously untested in the learned helplessness paradigm, were more similar to Holtzman rats in showing poor escape performance. However, of the three strains, Holtzman rats had the longest

escape latencies and the highest percentage of failed trials. Although there was a general trend for reduced activity of Holtzman rats, none of the individual measures of general activity were significantly different between all strains. Long-Evans rats showed longer rearing durations compared to Sprague Dawley rats, but not Holtzman rats. In general, however, strain differences in open-field behavior were minimal and could not account for the strain differences in escape latencies. This suggests that strain differences in escape latencies are due to an inherent susceptibility or resistance to helpless behavior that is not related to baseline locomotor behavior.

2.4.2 Holtzman rats appear helpless even in FR1 trials

Surprisingly, naïve Holtzman rats showed poor escape performance equal to that of inescapably shocked rats when the operant response required them to run out of the start compartment and back into it: a fixed ratio of 2 (FR2) schedule, which is generally considered necessary to demonstrate learned helplessness in rats (Grahn *et al.*, 2000; Hunziker & Dos Santos, 2007; Kram *et al.*, 2000). However, prior shock did significantly increase escape latencies for the FR1 trials. Indeed, 100% of naïve Holtzman rats were successful with the FR1 contingency, whereas a full one-third of inescapably shocked Holtzman rats failed this task, which is generally considered too easy for demonstrating learned helplessness in rats (Hunziker & Dos Santos, 2007). This finding cannot be due to an anomaly with our apparatus or task requirements since Sprague Dawley rats performed both FR1 and FR2 schedules with great ease. Rather, Holtzman rats appear to have such a strong predisposition to become immobile in response to stress that even naïve animals give up trying to escape under the added burden of an FR2 contingency.

Although we cannot rule out strain differences in pain processing as a contributor to the strain differences in escape behavior, this seems an unlikely explanation for several reasons. First, there is nothing in the literature to suggest that Holtzman rats

have altered pain sensitivity. For example, LaCroix-Fralish et al. reported no significant differences in painful responses to stimuli among Holtzman, Sprague Dawley, and Long-Evans male rats after lumbar root injury (LaCroix-Fralish *et al.*, 2005). Second, as indicated in our methods section, we tried several different shock intensities up to 1 mA, which did not alter escape performance, while 0.5 mA was sufficient to motivate FR1 escape performance in naïve rats. Finally, all rats consistently vocalized in response to shock, indicating significant emotional distress. Therefore, a lack of aversive motivation cannot explain the poor escape behavior of Holtzman rats.

We also cannot rule out that a general learning deficit associated with the Holtzman strain contributed to poor escape learning. However, naïve Holtzman rats are capable of achieving 100% efficiency when trained to press a lever to escape shock (Geller *et al.*, 1985) and they have superior acquisition of an avoidance response when compared to two other albino strains (Wistar and Sprague Dawley) (Kuribara *et al.*, 1976). Therefore, there is no evidence in the existing literature to suggest deficits in the Holtzman strain's pain perception or general learning ability. A more likely explanation for their poor escape performance is that the Holtzman strain is particularly predisposed to show hypoactivity as a consequence of stress (Spivey *et al.*, 2008). Indeed, we observed that many of the inescapably shocked rats reacted to the very first trial of escape learning with complete immobility. The operant response cannot be discovered if the rats stop moving when shocked.

2.4.3 Superior escape performance by females is explained by open-field activity

Like Dalla et al. (2008) we found that females appeared less helpless than males in terms of FR2 escape performance and that this sex difference appears independent of reproductive hormones, in that estrus cycle phase was unrelated to escape performance. However, the superior performance by females appears more related to

differences in baseline escape ability than to differences in learned-helplessness susceptibility. This is because there was no sex difference in FR1 escape latency, which was the schedule which showed an effect of learned helplessness training. Moreover, a difference in baseline activity level appears to underlie the sex difference. Females were more active than males in the open-field, and controlling for this activity difference eliminated the sex difference in FR2 escape performance. If increased locomotion in the open-field translates to increased locomotion in the shuttle box, this should give females a better chance to acquire the operant response and may explain their shorter escape latencies.

2.4.4 General activity and novelty reactivity are opposite predictors of helpless behavior

Based on activity differences observed in rats selectively bred for helpless behavior (Shumake *et al.*, 2005), we hypothesized that novelty reactivity (hyperactivity evoked by novel environments) would also confer greater risk for developing learned helplessness in a randomly bred population of rats. For the current study, we developed two classes of activity measures—general activity and novelty reactivity—and evaluated these measures as predictors of escape latency using linear regression. From Tables 2.3 and 2.4, one notices that the regression coefficients for general activity are universally negative while the regression coefficients for novelty-specific activity are, with one exception, positive. All but one general activity measure predicted significantly superior FR2 escape performance for females, explaining why controlling for general activity level eliminated the observed sex difference in FR2 escape behavior. Overall, open-field behavior accounted for 12% - 33% of the variance in FR1 escape latency (the contingency which showed an effect of inescapable shock), offering some support for generalized hyperactivity as a protective factor and novelty reactivity as a risk factor for

the development of helplessness. For males, a full third of the variance in escape behavior was explained by novelty reactivity. Such predictive trends in a general population could be magnified by selective breeding and thus give rise to the more dramatic novelty-reactivity differences previously observed in congenitally helpless rats (Shumake *et al.*, 2005).

It should be noted, however, that the literature is mixed with regard to whether novelty seeking is a marker of resilience or vulnerability to stress. In terms of the human literature, a study of Israeli undergraduates (Gil, 2005) found that decreased novelty-seeking (as determined by Cloninger's Tridimensional Personality Questionnaire, or TPQ; Cloninger) (Cloninger, 1987) predicted the development of PTSD after later exposure to a terrorist explosion. However, other studies using the TPQ found that combat veterans with PTSD showed greater novelty seeking (Wang *et al.*, 1997). High novelty seeking was also the strongest correlate of PTSD symptom severity in combat veterans (Richman & Frueh, 1997).

In terms of the animal literature, rats vulnerable to helplessness show an increased drive to explore novel environments (Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). However, Minor et al. (1994) also tested open-field behavior before helplessness training, and reported that neophobia or low novelty-seeking predicted vulnerability to helplessness. There are several differences between our study and that of Minor et al. including age of subjects, voluntary vs. involuntary access to the open field, and most importantly, the rat strain used. It is possible that the predictive relationship between novelty seeking and stress vulnerability may be reversed between "normal" and "extreme" populations, such as Sprague Dawley and Holtzman, respectively. This may also help explain the conflict in the human literature. For example, novelty seeking scores in combat veterans with PTSD (Wang *et al.*, 1997) were roughly twice those of

Israeli undergraduates with PTSD (Gil, 2005). Therefore the predictive relationship with novelty seeking may be different depending on the population, with combat veterans representing an extreme, similar to the congenitally helpless and Holtzman rats.

2.4.5 Potential biological mechanisms

Both the increased novelty seeking and the predisposition to developing helplessness seen in the Holtzman strain may be linked to abnormalities in glucocorticoid regulation. Congenitally helpless rats show elevated activity in the paraventricular nucleus of the hypothalamus (Shumake *et al.*, 2001), as do high novelty-responding rats (Kabbaj & Akil, 2001). Both congenitally helpless rats and high novelty responders also have reduced glucocorticoid receptor mRNA expression in the hippocampus (Kabbaj *et al.*, 2000; Lachman *et al.*, 1993), and low novelty responders can be converted into high responders with injection of a glucocorticoid antagonist into the hippocampus (Kabbaj *et al.*, 2000) – a manipulation that also turns otherwise resilient rats into helpless ones (Papolos *et al.*, 1993). In humans, lower cortisol levels predict higher novelty seeking scores in combat veterans with PTSD (Wang *et al.*, 1997).

Additionally, based on our metabolic brain mapping work with congenitally helpless and non-helpless rats (reviewed by Shumake and Gonzalez-Lima 2003), we may speculate that Holtzman and Sprague Dawley strains will show similar brain differences as these lines. Namely, Holtzman rats may show a hyperactive habenula coupled with hypoactive mesocorticolimbic dopamine system (Shumake & Gonzalez-Lima, 2003). Additionally, there may be a functional disconnection between forebrain and brainstem regions (Shumake *et al.*, 2004), leading to disinhibition and dysregulation of the stress response.

In summary, the Holtzman strain appears to offer a commercially-available model for studying susceptibility to helplessness and, translationally, to stress-related

psychopathology. Their strong predisposition toward passive coping should further allow investigators to utilize less shock at lower intensities along with simpler escape testing procedures (e.g. FR1 instead of FR2) to demonstrate learned helplessness, which would reduce experimental time and animal distress. Additionally, our results support Dalla et al. (2008) conclusion that the learned helplessness paradigm may be inadequate for modeling emotional disorders in females, and they offer a possible explanation for why this is the case. Namely, the baseline hyperactivity of some females might override or mask a state of helplessness, and escape behavior may not accurately reflect emotional disturbance in such hyperactive animals. In addition to the apparent protective effect of generalized hyperactivity in females, our results also offer some support that novelty-evoked hyperactivity confers greater risk for developing helpless behavior.

Chapter 3 Limbic-Cortical Network Differences between Responders and Non-responders to Fluoxetine Antidepressant Treatment in Rats

Neural network effects of antidepressant treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine were investigated using Holtzman rats. Animals underwent the forced swim test (FST) and immobility time was scored. On the next day, animals began receiving two weeks of fluoxetine (5 mg/kg) or vehicle and were retested in the FST at the end of treatment. Antidepressant behavioral effects of fluoxetine were analyzed using a ratio of immobility during pre- and post-treatment FST sessions. Brains were analyzed for regional metabolic activity and network interactions using quantitative cytochrome oxidase histochemistry and structural equation modeling. Fluoxetine exerted a protective effect against FST-induced immobility behavior in Holtzman rats. The mean regional metabolism of the nucleus accumbens shell differentiated fluoxetine-treated from vehicle-treated subjects, but not treatment responders from non-responders. The metabolic activities of infralimbic cortex and medial septum were predictive of antidepressant behavioral response, but these regions contributed opposite influences as evidenced by their opposite relationship to FST-induced immobility. A cortico-cortical correlogram revealed complex interactions among frontal cortex regions in fluoxetine responders that were less evident among non-responders and absent in the vehicle-treated group. Structural equation modeling of cortico-subcortical interactions revealed that direct path influences between the dorsal raphe nucleus and the lateral habenula and prelimbic cortex switched from negative to positive between fluoxetine-responders and non-responders, respectively. The observed differences in limbic-cortical interactions may represent an important neural network mechanism mediating the antidepressant SSRI response via modulation of the effective connectivity between the dorsal raphe and the lateral habenula and prelimbic cortex.

3.1 INTRODUCTION

In 2005, antidepressants surpassed antihypertensive agents to become the most commonly prescribed class of medications in office-based and hospital outpatient–based medical practice (Olfson & Marcus, 2009). However, not much is known regarding the neural mechanisms that underlie treatment response. Generally only 55 to 70 percent of patients respond to antidepressant treatment (Keller, 2005; Nelson *et al.*, 2008). As such, approximately 30 to 45 percent of patients are resistant to antidepressants. Depression and anxiety disorders such as post-traumatic stress disorder (PTSD) are most commonly treated with selective serotonin reuptake inhibitor (SSRI) antidepressants, of which fluoxetine is the prototypical drug (Devane *et al.*, 2005; Hemels *et al.*, 2002; Olfson & Marcus, 2009). Notably, Mayberg et al. (2000) reported that reciprocal changes in patterns of activity in the prefrontal cortex and subgenual cingulate (known as infralimbic cortex in rodents) could predict a response to fluoxetine in depressed patients. Increased knowledge of the inter-regional functional connections that are differentially affected in treatment responders and non-responders can aid in the understanding of neural network mechanisms underlying treatment resistance. In humans, PET and fMRI imaging are used to examine the effects of antidepressants but it is difficult to obtain optimal resolution of small subcortical structures, such as the habenula and dorsal raphe nuclei, which can also be part of a more complete functional network. Metabolic mapping techniques in animals are useful tools for studying the functional effects of antidepressant treatment because they have the spatial resolution to include individual subcortical nuclei.

In animals, the Porsolt forced swim test (FST) is used extensively as a model of behavioral despair (Porsolt *et al.*, 1978). In this model, rodents are subjected to inescapable stress, and depressive-like behavior is characterized by increased floating

behavior or immobility (Porsolt *et al.*, 1978). Treatment with standard antidepressant drugs can decrease FST immobility (Porsolt *et al.*, 1978; Cryan *et al.*, 2005b), an effect correlated with antidepressant efficacy in humans (Detke *et al.*, 1995; Porsolt *et al.*, 1977). Decreased immobility in the FST has correlated to treatment efficacy between different types of antidepressant drugs including fluoxetine (Porsolt *et al.*, 1978; Porsolt *et al.*, 1979). However, it is unknown whether the antidepressant-like effects of fluoxetine in the FST are correlated with changes in functional brain networks. The aim of this study was to investigate the interactions between anatomically and functionally related limbic and cortical regions implicated in the responsiveness to antidepressant treatment. This was achieved using quantitative cytochrome oxidase histochemistry and structural equation modeling. Cytochrome oxidase is the terminal respiratory enzyme in the mitochondrial electron transport chain that is correlated to ATP synthesis and serves as an endogenous metabolic marker for neuronal functional activity (Wong-Riley, 1989). In addition, cytochrome oxidase is a long-term indicator of brain metabolic capacity (Wong-Riley *et al.*, 1998), suggesting that histochemical quantification of cytochrome oxidase activity an ideal marker for examining the long-lasting effects of antidepressants on brain metabolism (Gonzalez-Lima & Cada, 1998; Gonzalez-Pardo *et al.*, 2008).

Functional network differences between fluoxetine responders, non-responders, and vehicle-controls were examined using interregional brain activity correlations and causal structural equation modeling (SEM). SEM is a multivariate modeling technique first applied to investigate brain network interactions by McIntosh and Gonzalez-Lima (1991, 1992, 1993, 1994, and 1995). SEM has been used to analyze neural network interactions using FDG, fMRI and cytochrome oxidase data (Bruchey *et al.*, 2007; Buchel & Friston, 1997; McIntosh & Gonzalez-Lima, 1991; McIntosh & Gonzalez-Lima, 1992; McIntosh & Gonzalez-Lima, 1993; McIntosh & Gonzalez-Lima, 1994; McIntosh &

Gonzalez-Lima, 1995; Puga *et al.*, 2007; Seminowicz *et al.*, 2004). Using cytochrome oxidase activity data, the SEM approach is suited for analysis of stable network interactions because cytochrome oxidase histochemistry maps the cumulative endpoint of long-term neuronal enzymatic activity (Gonzalez-Lima & Cada, 1998). This is the first study to describe the neural network effects of antidepressant treatment between fluoxetine-responders versus non-responders in an animal model.

3.2 MATERIALS AND METHODS

3.2.1 Subjects

Subjects were 35 male Holtzman rats from Harlan (Madison, WI) housed 2-3 per cage and maintained on a 12h/12h light/dark photoperiod in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Experiments occurred during the light phase between 0700 h and 1900 h. Food and water were available *ad libitum*. Prior to the start of experiments, subjects were handled and weighed for five minutes every day for one week. Tissue homogenate standards were obtained from a separate group of 20 adult Holtzman rats. Procedures were done in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee.

3.2.2 Behavior

Behavioral procedures are outlined in Table 3.1. Animals were evaluated for depressive-like behavior with the FST described by Porsolt *et al*. (1977). The FST is a 2-day procedure in which rats swim under conditions in which they cannot escape. Rats initially struggle to escape from the water, but eventually adopt a posture of immobility. When subjects are retested 24 hours later, immobility is increased.

On the first day of the FST, rats were placed in a clear acrylic cylinder (50.8 cm in height by 21.6 cm in diameter) filled to 37.1 cm with 25°C water. After 15 min of forced swimming (FST Day 1), the rats were removed from the water, dried with towels, and placed in a cage on top of a heating pad for 30 min. The cylinders were emptied and cleaned between rats. At 24 hours after the forced swim, rats were retested for 5 min under identical swim conditions (FST Day 2). Both forced swimming sessions were videotaped from the side of the cylinders and scored by raters unaware of the treatment condition. Immobility time was defined as the time during which the rat remained in a stationary posture that did not reflect attempts to escape from the water. In this characteristic posture, the forelimbs are motionless and tucked toward the body. A rat was judged to be immobile if it was making only movements necessary to keep its head above water.

Table 3.1 Experimental Design

Method	Approximate Age
Handle (one week)	PD 40-49
Pre-treatment FST (2 Days)	PD 50-51
Treatment (IP FLX or VEH, 2 weeks)	PD 52-66
Post-treatment FST (2 Days)	PD 66-67
Tissue processing	PD 68

FST, Forced swim test. FLX, Fluoxetine. VEH, Vehicle. PD, Postnatal day.

3.2.3 Drugs

Fluoxetine hydrochloride (Spectrum Chemicals, Gardena, CA) was reconstituted using a dimethylsulfoxide (DMSO) / normal saline vehicle (25/75 percent) to make a 2.5 mg/ml fluoxetine solution. Fluoxetine (5 mg/kg) or vehicle (25% DMSO / 75% normal saline) was administered intraperitoneally (i.p.) once a day for two weeks beginning one day after the baseline FST Day 2. We chose to study chronic effects because the therapeutic response to antidepressant drugs in depressed humans does not occur after acute administration, but rather emerges slowly over the course of weeks. Although acute administration of SSRIs can improve FST performance, this occurs at high doses (≥ 20 mg/kg) (Bianchi *et al.*, 2002; Dow *et al.*, 2005; Jang *et al.*, 2009) that are well above clinically effective doses for humans. However, Detke et al. (1997) showed that a lower dose of fluoxetine (5 mg/kg) is effective in the FST when given chronically (daily injections for 2 weeks). On PD 66, animals were retrained in the forced swim cylinder for 15 min and then received the last fluoxetine dose. Twenty-four hours later, they were re-tested in the FST for 5 min.

3.2.4 Histochemistry

Following decapitation, brains were removed intact and frozen rapidly in isopentane. Using a cryostat (Reichert-Jung) at -20°C, brains were sectioned at 40 µm and kept frozen at -40°C until they were processed using quantitative cytochrome oxidase histochemistry, as described previously (Gonzalez-Lima & Cada, 1998). Briefly, the procedure involves a series of chemical exposures, the first of which facilitates tissue adherence to the slides (0.1 M phosphate buffer with 10% wt/vol sucrose and 0.5% vol/vol glutaraldehyde, pH 7.6, for 5 min). The next series of exposures (three changes of 0.1 M phosphate buffer with 10% wt/vol sucrose, for 5 min each) removes red blood

cells. Then slides undergo metal intensification (0.05 M Tris buffer, pH 7.6, with 275 mg/l cobalt chloride, 10% wt/vol sucrose, and 0.5% vol/vol dimethylsulfoxide, for 10 min) followed by incubation to stain for cytochrome oxidase (350 mg diaminobenzidine tetrahydrochloride, 52.5 mg cytochrome c, 35 g sucrose, 14 mg catalase, and 1.75 ml dimethylsulfoxide in 700 ml of oxygen-saturated 0.1 M phosphate buffer, at 37°C for 1 h). The reaction is stopped by fixing the tissue (30 min at room temperature with 10% wt/vol sucrose and 4% vol/vol formalin). Finally, slides are dehydrated in ethanol baths (increasing from 30% to 100% vol/vol ethanol), cleared with xylene, and coverslipped with Permount.

3.2.5 Preparation of standards and densitometric analysis

To quantify enzymatic activity and to control for staining variability across different batches of cytochrome oxidase staining, sets of tissue-homogenate standards were included with each batch of slides. The enzymatic activity of cytochrome oxidase in this homogenate was assayed using spectrophotometry, as described by Gonzalez-Lima and Cada (1998), and activity units were defined at pH 7 and 37°C, where 1 unit oxidizes 1 µmol of reduced cytochrome c per min (µmol/min/g tissue wet weight). Immediately prior to each staining procedure, the standard homogenate was sectioned at varying thickness (10, 20, 40, 60, and 80 µm), thaw-mounted onto a slide, and stained for cytochrome oxidase along with the experimental tissue. Activity values from the spectrophotometry were assigned to each standard, and calibrated to corresponding optical density measurements of the sections. The resulting linear regression equations ($r^2 > 0.90$) were used to convert optical density readings from brain regions of interest into cytochrome oxidase activity values.

Using an image-processing system (JAVA, Jandel Scientific, Corte Madera, CA), optical density was sampled from 33 regions selected based on previous research by our lab (Shumake *et al.*, 2000; Shumake *et al.*, 2004; Shumake & Gonzalez-Lima, 2003) and neuroimaging studies showing treatment effects (Freo *et al.*, 2008; Mayberg *et al.*, 1999; Mayberg *et al.*, 2000) (Figure 3.1). Cytochrome oxidase-stained frontal cortical regions show differences in labeling between the outer half (up to layer III) and the inner half and thus both superior and deep cortical layers were analyzed separately. The regions included were the prelimbic (PL), dorsal frontal (DF), lateral frontal (LF), anterior insular (AI), lateral orbital (LO), medial orbital (MO), infralimbic (IL), anterior cingulate (CG), lateral septum (LS), medial septum (MS), nucleus accumbens (Acb) shell and core, ventral pallidum (VP), medial habenula (MH), lateral habenula (LH), subiculum (S) dorsal and ventral, dentate gyrus (DG), hippocampus (CA1 and CA2), retrosplenial cortex (RS), periaqueductal gray (PAG), interpeduncular nucleus (IP), ventral tegmental area (VTA), and dorsal raphe nucleus (DR). The sampling window was adjusted for each region so that it was as large as possible while still allowing for four, non-overlapping readings to be taken bilaterally from each region. In addition, each region was sampled from three different sections per animal, and these readings were averaged in order to obtain representative values for each region for each subject.

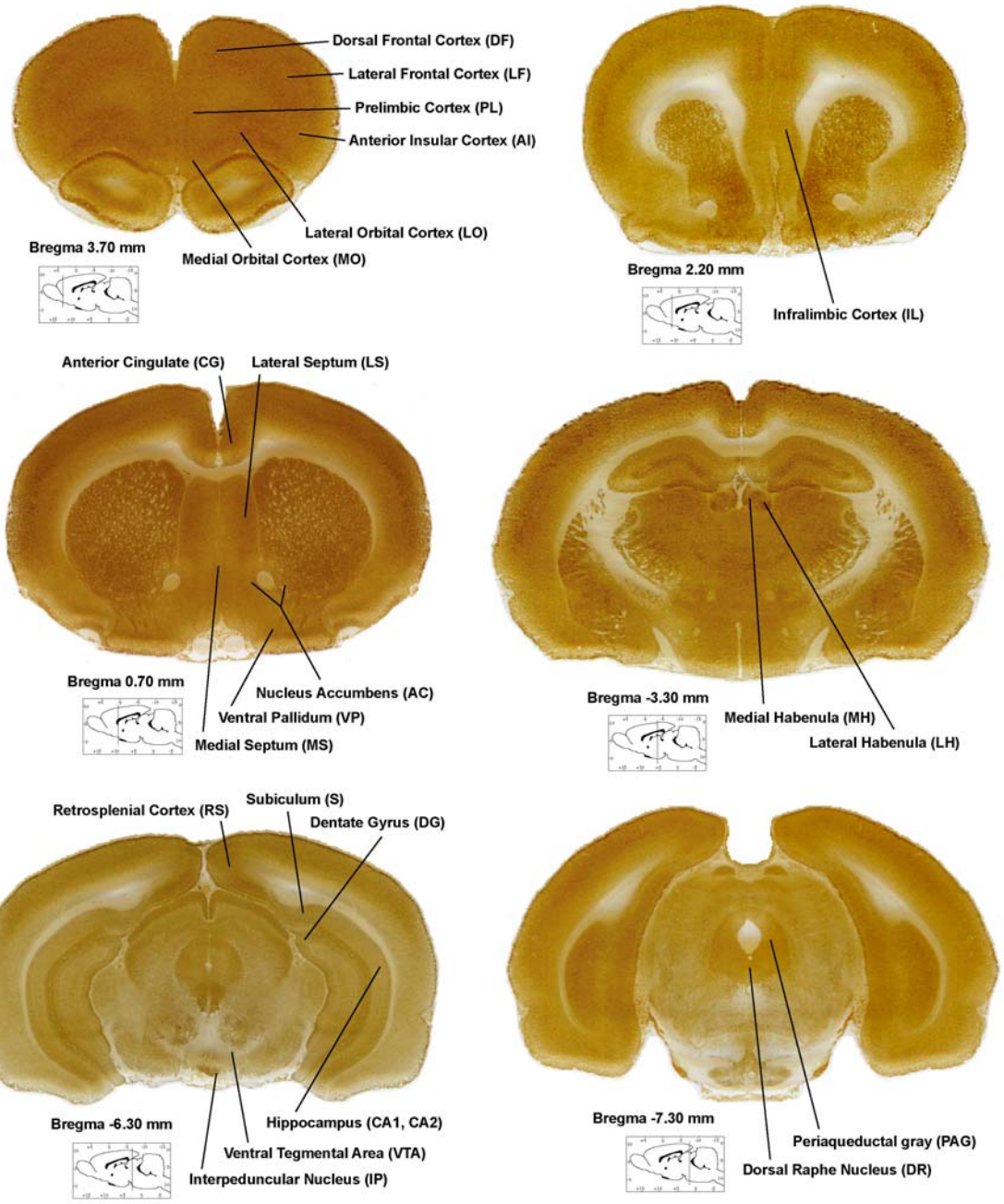

Figure 3.1 Coronal brain diagrams. Each image is a coronal cytochrome oxidase-stained section depicting regions of interest by Bregma level. Anterior–posterior Bregma coordinates are indicated below each diagram.

3.2.6 Statistical Analysis

A ratio of immobility time during pre- and post-treatment FST Day 2 sessions was calculated and termed the FST treatment response ratio. This ratio standardized differences in immobility that were present before treatment. Two-step clustering analysis was applied to identify how subjects' FST treatment response ratio differentiated them into distinct groups or clusters (Chourbaji *et al.*, 2005). This analysis calculated the dissimilarity or the distance between individuals based on their FST immobility, and segregated into two clusters: animals that responded to treatment and those that did not respond.

Regional mean differences in cytochrome oxidase activity between responders, non-responders and vehicle were evaluated using one-way ANOVA. Two-tailed Pearson correlations were also performed between regional brain cytochrome oxidase activity and forced swim behavior.

Pearson product-moment correlations between all measured regions were calculated for each group (within-group analysis). A jackknife procedure was performed in which each individual subject was dropped from a group, and then correlations were calculated again without that subject's data. This procedure was iterated until each subject had been sequentially dropped and the analysis repeated. To minimize Type I error, correlations that remained significantly different from zero at $p < 0.05$ through all iterations were considered statistically reliable for further analysis. The Fisher Z transformation was used to convert each correlation to a Z score to test differences in regional correlations between groups (between-group analysis) (Bruchey & Gonzalez-Lima, 2006; Jones & Gonzalez-Lima, 2001; Puga *et al.*, 2007). The term functional connection or functional coupling is used to refer to a significant correlation between two

brain regions. Based on data from the jackknife correlations, a color correlogram was developed using Matlab software version 7.0 (Nair *et al.*, 2001).

Regions that had significantly different correlations between groups were selected for path analysis or structural equation modeling (SEM) to investigate the effective connectivity underlying the response and non-response to fluoxetine. Models were created using the LISREL software package version 8.54. In SEM of cytochrome oxidase data, the functional influences between anatomically-connected regions are given a numeric weight, or path coefficient, derived through a process of iterative data fitting (Puga *et al.*, 2007). Specifically, path coefficients represent the proportion of cytochrome oxidase activity in one region determined by the cytochrome oxidase activity of other areas that directly project to that region. These directional paths quantify the causal influences between regions and how they are modified between different conditions. Path coefficients greater than 1 were fixed at 0.96 to improve model stability (McIntosh & Gonzalez-Lima, 1994; Seminowicz *et al.*, 2004).

3.3 RESULTS

Data analysis was performed in two steps. First, we compared the fluoxetine versus vehicle groups in terms of their FST immobility and the effects of drug on brain metabolism. Second, fluoxetine-treated subjects were classified into responders and non-responders based on a two-step cluster analysis of their FST treatment response ratio. This was followed by comparisons of FST immobility and patterns of brain metabolism between fluoxetine responders, non-responders and vehicle-treated controls.

3.3.1 Fluoxetine versus Vehicle

3.3.1.1 Confirmation of Fluoxetine Antidepressant Behavioral Effects on Holtzman Rats

The immobility of rats in the FST showed evidence of antidepressant effects after two weeks of fluoxetine treatment. A ratio of pre- and post-treatment FST Day 2 immobility was computed and termed the FST treatment response ratio, with higher values indicating a greater therapeutic response to fluoxetine (i.e. reduction of immobility in the post-treatment session). The FST response ratios were normally distributed; therefore, a univariate ANOVA was performed with the FST response ratio as the dependent variable and treatment as the independent variable. One subject was an outlier (defined as more than 3 standard deviations over the mean) and was excluded from the analysis, resulting in a total of 34 subjects. There was a treatment effect in which fluoxetine significantly increased the FST response ratio ($p = 0.003$) (Table 3.2). Another ratio of pre- and post-treatment FST Day 1 immobility was also assessed; however there were no significant differences between groups in this measure ($p = 0.80$).

Table 3.2 Means analysis of immobility in the FST Day 2 between fluoxetine and vehicle groups

FST 5-min session	Fluoxetine (n=18)	Vehicle (n=16)
	Mean ± SE	Mean ± SE
Immobility pre-treatment (sec)	78.6 ± 9.9	61.5 ± 10.2
Immobility post-treatment (sec)	62.9 ± 7.4	71.9 ± 7.5
Immobility ratio pre/post-treatment	1.26 ± 0.09 *	0.83 ± 0.11

* $p < 0.01$, fluoxetine versus vehicle.

3.3.1.2 Fluoxetine Reduced Mean Cytochrome Oxidase in the Nucleus Accumbens Shell

The only significant mean regional difference in cytochrome oxidase activity between fluoxetine-treated subjects and vehicle-treated subjects was in the shell of the nucleus accumbens ($p < 0.05$) (Figure 3.2). To determine whether fluoxetine treatment produced general effects on brain metabolism, the cytochrome oxidase measurements from all regions were averaged for fluoxetine and vehicle-treated groups. There was no significant difference on overall brain metabolic activity ($p = 0.70$).

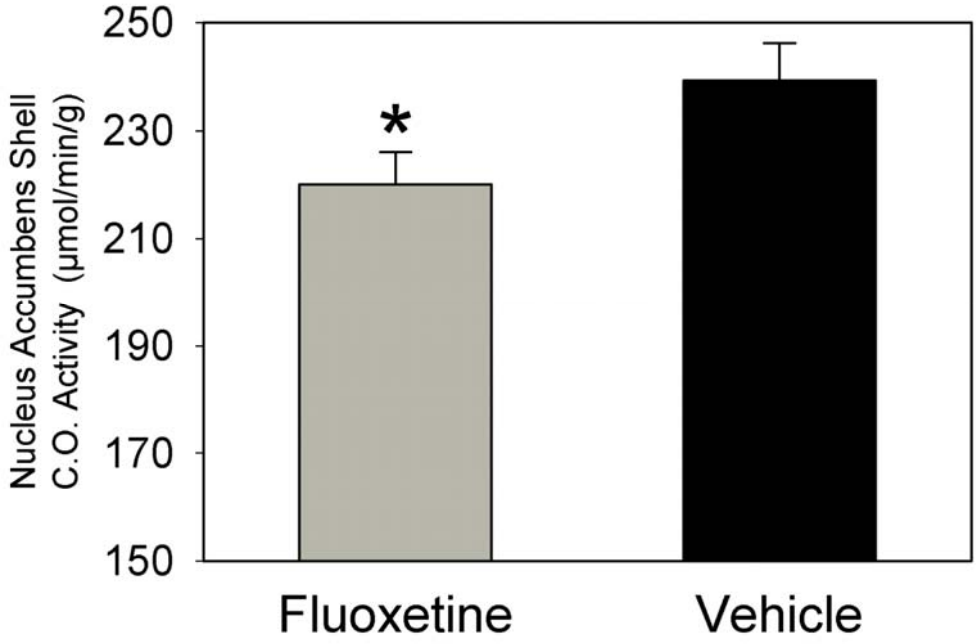

Figure 3.2 Fluoxetine reduced cytochrome oxidase (C.O.) activity in the shell of the nucleus accumbens. The fluoxetine group had mean cytochrome oxidase activity of 220 ± 6 and the vehicle had 239 ± 7 ($p < 0.05$).

3.3.2 Fluoxetine Responders versus Non-responders

3.3.2.1 Fluoxetine Produced Opposite Brain-Behavior Correlations in the Infralimbic Cortex and Medial Septum

Fluoxetine-treated subjects were classified into responders and non-responders based on a two-step cluster analysis of their FST treatment response ratio. As seen in Table 3.3, the non-responders showed a ratio very close to 1, indicating virtually no change in behavior as a result of fluoxetine treatment. Individual differences in the behavioral response of animals to the fluoxetine treatment allowed for the calculation of correlations between their behavioral response and the cytochrome oxidase activity of individual brain regions. Such brain-behavior correlations revealed that activity in the deep layers of the infralimbic cortex and the medial septum were negatively and positively correlated, respectively, with FST Day 2 immobility among fluoxetine-treated subjects (Figure 3.3). In addition, vehicle-treated subjects showed significant positive brain-behavior correlations with the FST treatment response ratio and the inner layers of the prelimbic and lateral orbital cortices ($r = .60$ and $.71$ respectively, $p < 0.05$). There were no other significant brain-behavior correlations.

Table 3.3 Cluster analysis of FST treatment response ratio

Cluster	N	Mean ± SE
Fluoxetine responders	7	1.63 ± 0.09 *
Fluoxetine non-responders	11	1.02 ± 0.06
Total	18	1.26 ± 0.09

* $p < 0.01$, responders versus non-responders.

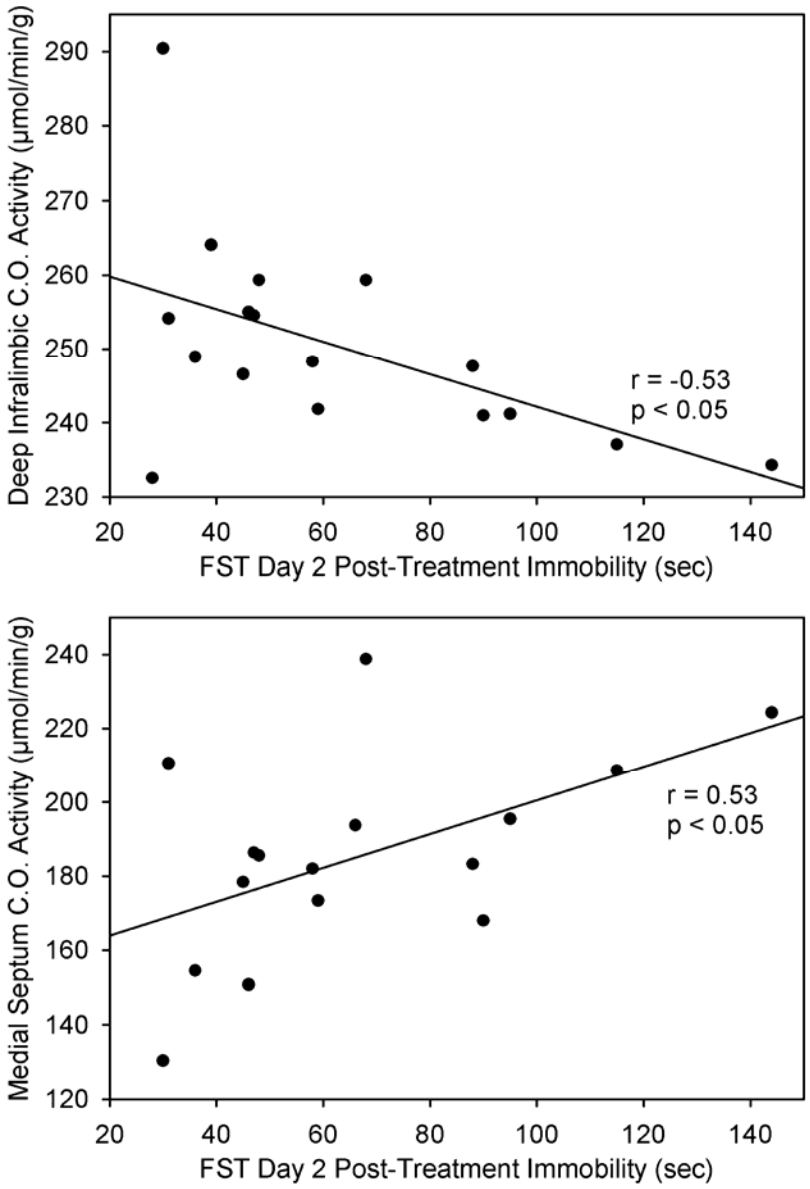

Figure 3.3 Top, FST post-treatment immobility was negatively correlated with cytochrome oxidase activity in the deep layers of the infralimbic cortex ($r = -0.53$, $p < 0.05$, $n = 17$). Bottom, FST post-treatment immobility was positively correlated with cytochrome oxidase activity in the medial septum ($r = 0.53$, $p < 0.05$, $n = 16$). Only fluoxetine-treated subjects are shown.

3.3.2.2 Cortico-Cortical Interactions Differentiated Between Vehicle-Treated, Fluoxetine Responders and Non-Responders

The large, rich datasets derived from the regional metabolic mapping allowed for the measurement of virtually all cortical and subcortical regions in fluoxetine-treated and vehicle-treated subjects. This afforded the capability to evaluate not only mean activity changes but also the covariance relationships between regions throughout the brain. Particularly striking were the observed patterns of inter-correlations among cortical regions in these subjects that are implicated in human depressive behavior. Figure 3.4 is a correlogram illustrating the patterns of inter-regional correlations between fluoxetine responders, non-responders and vehicle-treated subjects using the superficial layers of cortical regions. The superficial cortical layers were used for this analysis to focus on cortico-cortical projections. A greater number of red and blue squares was indicative of increased positive and negative correlations or functional couplings, respectively, between prefrontal superficial layers. There was a remarkable increase in complexity in the pattern of interactions between prefrontal regions in the responder group in comparison to the vehicle-treated group, which showed uniformly positive correlations in Figure 3.4 as indicated by all red squares. In addition, responders showed stronger functional correlations between prefrontal superficial regions compared to non-responders that showed functional de-couplings in general. For example, the dorsal frontal cortex in the responders was positively correlated with the prelimbic and lateral frontal cortices and the prelimbic cortex was positively correlated to the lateral orbital and negatively correlated to the insular cortex. The most significant finding was that the infralimbic cortex (IL at bottom of correlogram) changed from being highly positively correlated (red colors) with other prefrontal cortical regions (dorsal frontal, lateral frontal, anterior insular, lateral orbital, medial orbital, and prelimbic cortices) in the vehicle-

treated group to being uncorrelated in the non-responder group (green colors), whereas among responders the infralimbic cortex was negatively correlated (blue colors) to the dorsal frontal, lateral frontal, and insular cortices.

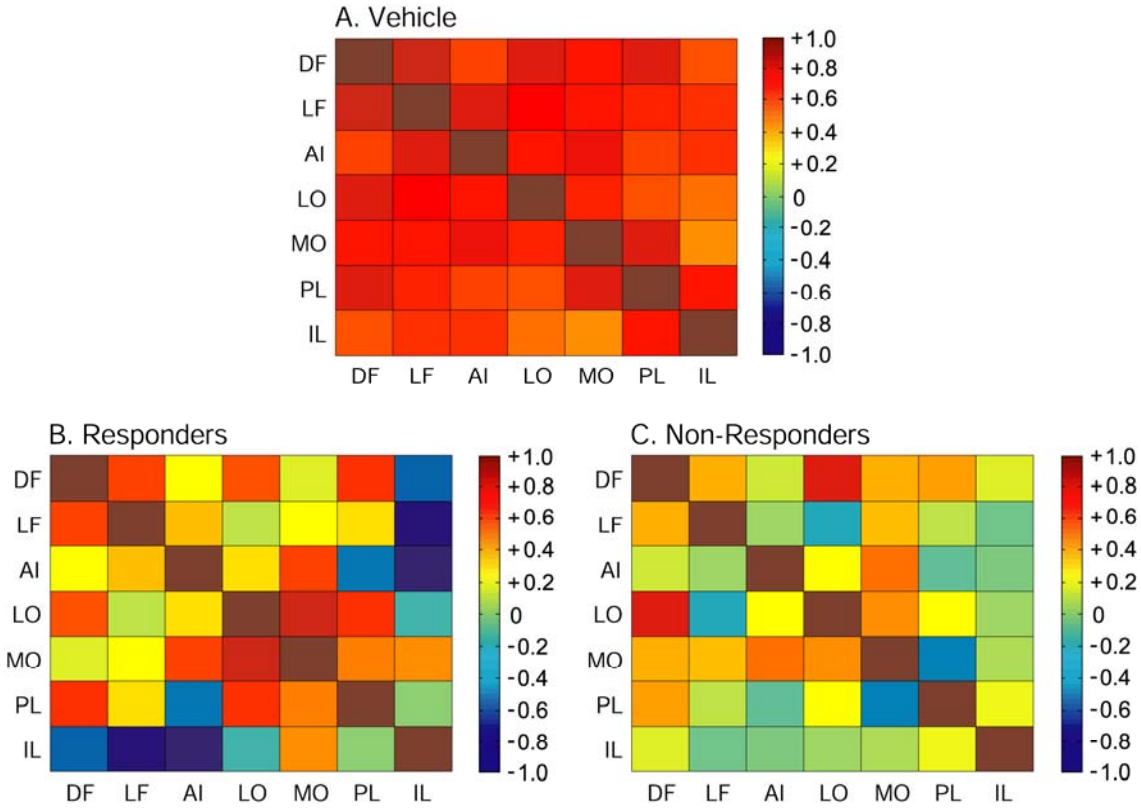

Figure 3.4 Correlogram of superficial layer cortical interactions. The scale shows the relationship between positive and negative correlations. Positive correlations are represented as red and negative correlations are blue. Darker colors signify higher correlations. Green colors represent correlations equal to or approximating zero. Only superficial cortical layers are depicted since we are focusing on cortical-cortical projections. DF (dorsal frontal), LF (lateral frontal), AI (anterior insular), LO (lateral orbital), MO (medial orbital), PL (prelimbic) and IL (infralimbic cortex).

3.3.2.3 Subcortical Interactions Implicated the Dorsal Raphe, Ventral Tegmental Area, Periaqueductal Gray and Habenulointerpeduncular Pathway

Using a hypothesis-driven approach to identify significant pairwise correlations can become complicated with large datasets derived from regional metabolic mapping. Therefore, a data-driven approach was used to identify, without *a priori* hypotheses, the nature of the fluoxetine effects in an unbiased manner. The within-group analysis showed that in each group there were regional pairwise inter-correlations that remained significantly different from zero after all jackknife combinations. The vehicle, responder and non-responder within-group data are shown in three cross-correlations matrices (Tables 3.4, 3.5, and 3.6). In addition to the superficial cortical layers analysis, responders showed strong positive correlations between the deep dorsal frontal cortex and the prelimbic and deep medial orbital cortices as opposed to non-responders which showed weaker negative correlations. Interestingly, the prelimbic cortex was correlated with the key subcortical regions that differentiated the groups.

The cross-correlations between-groups analysis yielded major differences in group comparisons. The main significant group differences can be summarized as follows:

1) The dorsal raphe was negatively correlated to the prelimbic cortex and habenular nuclei in the responder group as opposed to the positive correlations observed among non-responders.

2) The superficial prelimbic cortex had a low positive correlation with the ventral tegmental area among responders and this pair of regions had a strong negative correlation in the non-responder group.

3) The prelimbic, dorsal frontal, and lateral orbital cortices were strongly negatively correlated with the periaqueductal gray among responders and these regions showed low positive correlations or were not correlated among non-responders.

4) The dorsal raphe was positively correlated with the medial habenula, lateral habenula and interpeduncular nucleus among non-responders and it showed negative correlations with the habenula and was not correlated to the interpeduncular nucleus among responders.

5) The dorsal raphe was strongly correlated with orbital, prelimbic and deep lateral frontal cortices among responders and was not correlated with these regions in the vehicle group.

Table 3.4 Pairwise Pearson correlations in the vehicle group

	DF s	DF d	LF s	LF d	AI s	AI d	LO s	LO d	MO s	MO d	PL s	PL d	IL s	IL d	CG s	CG d	DR	MH	LH	IP
DF s	1.00																			
DF d	.93	1.00																		
LF s	.84	**.79***	1.00																	
LF d	.81	**.78***	**.97***	1.00																
AI s	.60	**.69^**	**.79***	**.81^***	1.00															
AI d	.39	.52	.73	**.77***	**.90***	1.00														
LO s	.81	.75	**.74***	.73	.70	.50	1.00													
LO d	.82	.88	**.85***	.87	.68	.64	.70	1.00												
MO s	.72	.81	.69	.63	.77	.65	.68	.67	1.00											
MO d	.81	**.90***	.66	.60	.57	.41	.67	.71	**.86***	1.00										
PL s	.78	**.87***	.67	.65	.60	**.47^**	.57	.64	**.80***	**.91***	1.00									
PL d	.56	**.72***	.65	.66	**.73^**	.69	.43	**.57^**	**.74***	.71	.85	1.00								
IL s	**.58^**	**.60^**	**.66^**	**.72^***	**.63^**	**.56^**	.53	.42	.45	.54	.71	.69	1.00							
IL d	**.80***	**.73^***	**.73***	**.77^***	**.58^**	**.41^**	.66	.55	**.48^**	.60	.69	.57	.87	1.00						
CG s	-.02	-.03	.15	.08	.11	.31	.00	.23	.12	**.00***	.00	.10	-.01	-.04	1.00					
CG d	-.02	-.01	.08	.01	-.02	.20	.02	.19	.04	**.01***	.00	.04	-.02	-.10	.93	1.00				
DR	.01	.05	-.02	**.00^**	-.04	.00	**.00^**	**-.04^**	**-.02^**	**-.03^**	**-.01^**	**.02^***	-.01	.01	.01	.02	1.00			
MH	.01	.01	**-.05***	.00	.02	.00	.07	-.03	.01	**-.01***	.03	.00	.19	.05	-.07	-.14	**-.60***	1.00		
LH	-.33	-.15	**-.32***	**-.16***	-.09	**-.07***	-.17	-.26	-.26	-.26	-.09	-.08	-.03	.03	.00	**-.01^**	**-.88***	.75	1.00	
IP	.14	.03	.00	-.03	.00	-.14	**-.04***	.11	.10	.11	**-.02^**	-.02	.03	.11	-.16	-.16	**.00***	**-.14***	**-.45***	1.00
AC s	**-.03***	**-.01***	.02	.00	.00	-.01	.08	**.05***	.06	.00	-.01	-.04	-.03	.05	.65	.54	-.18	-.03	.00	-.06
AC c	**-.03***	**.13***	.12	-.05	.22	.24	**-.03***	**.04***	.19	.01	.14	.26	.00	-.01	.68	.55	-.08	-.01	.00	-.18
PAG	**-.06^**	**-.25^**	.01	-.12	-.34	-.18	**-.25^**	**-.05^**	-.11	-.26	**-.23^**	**-.16^**	.08	.14	-.02	.01	.61	**-.80***	**-.61***	-.05
RS	.14	.14	.00	.02	.02	.02	.03	-.03	-.07	.18	.23	.06	.04	.00	.06	.01	-.07	**.03***	**-.02***	.06
VTA	.02	-.12	-.04	**.01^**	.00	-.01	.01	**.03***	-.36	-.02	**-.35***	-.34	.04	-.11	.44	.41	.02	.02	.08	-.17

***Vehicle vs. non-responder, p < 0.05; ^Vehicle vs. responder, p < 0.05. s, superficial layer; d, deep layer. DF, dorsal frontal cortex. LF, lateral frontal cortex. AI, anterior insular cortex. LO, lateral orbital cortex. MO, medial orbital cortex. PL, prelimbic cortex. IL, infralimbic cortex. CG, cingulate cortex. DR, dorsal raphe nucleus. MH, medial habenula. LH, lateral habenula. IP, interpeduncular nucleus. AC s, nucleus accumbens shell. AC c, nucleus accumbens core. RS, retrosplenial cortex. VTA, ventral tegmental area.**

Table 3.5 Pairwise Pearson correlations in the responder group

	DF s	DF d	LF s	LF d	AI s	AI d	LO s	LO d	MO s	MO d	PL s	PL d	IL s	IL d	CG s	CG d	DR	MH	LH	IP
DF s	1.00																			
DF d	.91	1.00																		
LF s	.61	.61	1.00																	
LF d	-.04	.29	.50	1.00																
AI s	.24	**-.39***	.34	**-.60***	1.00															
AI d	-.15	-.18	.09	.06	.53	1.00														
LO s	.58	.76	.11	.47	.30	.20	1.00													
LO d	.68	.89	.19	.37	.01	-.17	.93	1.00												
MO s	.17	.40	.23	.31	.62	.03	.84	.77	1.00											
MO d	.53	**.79^**	.06	.13	-.17	.39	.68	.81	.56	1.00										
PL s	.65	**.88^**	.30	.44	-.38	**-.58***	.64	.88	.49	**.94^**	1.00									
PL d	.61	**.88^**	.37	-.59	**-.20***	-.23	**.85^**	**.95^***	.61	.79	.93	1.00								
IL s	**-.47***	**-.45***	**-.84***	**-.52***	**-.90***	**-.99^***	-.13	-.05	.44	.26	.02	-.19	1.00							
IL d	.12	**-.29***	-.15	**-.39***	**-.91***	**-.74***	.00	-.08	**-.56***	-.38	-.15	.06	.35	1.00						
CG s	.03	-.12	-.05	-.05	.00	-.04	-.11	.02	-.18	-.58	-.45	-.20	.29	-.06	1.00					
CG d	-.23	-.41	-.18	.21	-.19	-.26	-.10	-.26	.02	-.77	-.68	-.45	-.23	-.10	.78	1.00				
DR	-.68	-.69	.24	**.98^***	-.65	.44	**-.89^***	**-.92^***	**-.99^***	**-.99^***	**-.95^***	**-.86^***	-.09	.46	.00	.45	1.00			
MH	-.47	.54	**-.02^**	-.20	.03	-.01	.39	.60	.45	-.38	-.49	.40	.03	-.26	.05	-.27	**-.55^**	1.00		
LH	.16	.25	.12	.02	-.08	**.02^**	-.15	.02	-.29	.59	**.49^**	.21	.06	-.24	-.50	**-.84***	**-.64^**	.72	1.00	
IP	.52	.55	.38	-.03	.07	.17	.26	-.09	.00	.78	**.89***	.39	-.02	.26	.24	.06	**-.05^**	.51	.38	1.00
AC s	**-.03^**	-.07	.09	.00	.16	-.23	-.01	**-.06^**	.10	-.18	-.09	.26	.02	.59	.06	.48	.05	-.31	-.49	-.02
AC c	**.67^**	**.64^**	.50	-.29	.15	.64	**.78^**	**.59^**	.38	.19	.25	.59	-.04	-.07	-.04	-.02	-.36	-.03	.01	-.05
PAG	**-.98^***	**-.98^***	-.37	.67	-.07	-.17	**-.99^***	**-.97^***	-.73	-.85	**-.95^***	**-.99^***	.42	.43	-.18	-.25	.72	-.46	-.33	-.19
RS	-.05	.10	.18	.50	-.02	.06	.04	.07	.01	.02	.16	-.08	-.72	-.36	-.17	-.08	-.06	.01	-.04	.04
VTA	-.02	.31	.45	**.95^***	-.37	.37	.50	**.40^**	.33	.11	**.41^**	-.66	-.40	-.23	-.02	.12	-.38	-.18	.00	-.40

***Responder vs. vehicle, p < 0.05; ^Responder vs. non-responder, p < 0.05**

Table 3.6 Pairwise Pearson correlations in the non-responder group

	DF s	DF d	LF s	LF d	AI s	AI d	LO s	LO d	MO s	MO d	PL s	PL d	IL s	IL d	CG s	CG d	DR	MH	LH	IP
DF s	1.00																			
DF d	.78	1.00																		
LF s	.40	**.03***	1.00																	
LF d	.36	**-.12***	.22	1.00																
AI s	.14	.42	**.03***	**-.14***	1.00															
AI d	-.11	.00	.47	**-.37***	**-.13***	1.00														
LO s	.79	.65	**-.19***	.11	.24	.43	1.00													
LO d	.91	.80	**-.37***	.47	.44	.19	.81	1.00												
MO s	.40	.28	.35	.38	.53	.31	.45	.69	1.00											
MO d	.26	**-.35^***	.52	-.11	.01	-.14	.29	.11	**.18***	1.00										
PL s	.42	**-.58^***	.12	.80	-.09	-.08	.23	.57	**-.37***	**.13^***	1.00									
PL d	.21	**-.09^***	.45	.46	.07	.12	**.05^**	**.37^**	**.00***	.43	.54	1.00								
IL s	.18	.00	-.05	**.00***	-.01	**.08^**	.04	.17	.08	.56	.19	-.01	1.00							
IL d	**-.01***	**-.01***	**.01***	**.01***	.00	.08	.00	-.06	-.09	.25	.15	.00	.69	1.00						
CG s	-.17	-.20	-.26	-.09	.07	-.21	.11	-.15	-.07	**-.85***	-.29	-.34	-.02	-.16	1.00					
CG d	.01	-.14	-.21	-.04	-.04	-.28	.12	-.06	.05	**-.85***	-.42	-.34	-.04	.28	.95	1.00				
DR	-.01	-.06	.64	**.09^**	.01	.80	**-.08^**	**-.09^**	**.30^**	**.26^**	**.62^**	**.77^***	-.02	.53	.08	.14	1.00			
MH	.26	.54	**.99^***	-.09	.06	.33	.05	.44	.57	**-.81***	.21	.25	.24	.33	-.15	-.09	**.74^***	1.00		
LH	-.09	-.37	**.67***	**.81***	.06	**.93***	.03	-.12	-.51	-.09	**-.60^**	-.57	.22	.17	-.27	-.25	**.80^***	.93	1.00	
IP	-.04	-.08	.02	.05	-.07	.31	**.86***	.55	.26	-.13	.50	.03	.10	.02	.15	.03	**.87^***	**.66***	**.74***	1.00
AC s	**-.99***	**-.78***	-.28	-.14	-.25	-.30	-.61	**-.86^***	-.43	.13	-.28	-.26	.01	-.01	.37	.20	-.03	.07	.00	.05
AC c	**-.81***	**-.95***	-.21	-.31	-.53	-.22	**-.84^***	**-.85^***	-.35	-.30	-.36	.11	.14	.27	.08	-.04	.00	-.04	-.06	.07
PAG	**.14^**	**.37^**	.41	.05	-.06	-.01	**-.06^**	**.21^**	.16	-.11	**.37^**	**.26^**	.39	-.23	-.06	.01	-.03	**.19***	**.18***	-.19
RS	-.07	-.19	.00	.00	.00	.39	.06	.00	.21	.24	.13	.02	.25	.37	.03	-.01	.65	**.79***	**.74***	.53
VTA	.00	.02	.06	**-.30^**	-.42	-.12	-.11	**-.96^***	-.30	.17	**-.89^***	-.30	.02	.00	.04	.02	.00	-.28	-.35	-.10

***Non-responder vs. vehicle, p < 0.05; ^Non-responder vs. responder, p < 0.05**

3.3.2.4 Structural Equation Modeling of Cortico-Subcortical Effective Connectivity Implicated Prelimbic, Dorsal Raphe and Habenulointerpeduncular Pathways

To further understand the functional interrelationships between cortical and subcortical regions across anatomically defined paths, bivariate correlations from the previous analysis were used to compute path coefficients between regions and groups in a data-driven SEM network. The final model consisted of the prelimbic deep cortical layers, dorsal raphe nucleus, lateral and medial habenula, interpeduncular nucleus, and their major anatomical connections (Figure 3.5). Regions or nodes were selected based on the correlations that were significantly different between treatment groups. Paths between these regions were based on known anatomical connections. The prelimbic cortex sends projections to the dorsal raphe and lateral habenula (Vertes, 2004). The dorsal raphe projects to the habenula and the medial prefrontal or prelimbic cortex (Lowry *et al.*, 2008). The lateral habenula sends projections to the dorsal raphe (Aghajanian & Wang, 1977). The medial habenula sends most of its fibers to the interpeduncular nucleus (Contestabile & Flumerfelt, 1981; Herkenham & Nauta, 1979) and also sends axonal projections to the lateral habenula (Kim & Chang, 2005). Finally, the interpeduncular nucleus sends projections to the dorsal raphe (Shibata & Suzuki, 1984).

The analysis included regions of interest that were combined for simplicity to compute causal influences between regions using SEM. The initial model was not solvable, due to the existence of reciprocal connections. This model was modified until a final "best" model was identified. Residuals on nodes were constrained to 35% so that most of the variance was accounted for from within the model (Seminowicz *et al.*, 2004). Chi-square analysis revealed that a network model where all paths were free to vary was significantly different from a model with fixed paths (chi-square difference = 28.28, $p < 0.01$). This indicated that the patterns of interactions among the selected regions were

statistically different between the responder, non-responder and vehicle-treated groups. Figure 3.5 illustrates the differences in path coefficients between vehicle, responder, and non-responders groups. The values for direct and total effects of the regions among vehicle, responder and non-responder groups are depicted in Table 3.7. The direct effects represent the direct influence or path coefficient of one region on another. The total effects represent the sum of direct and indirect effects of a region on another region. For example, the interpeduncular nucleus can indirectly affect the lateral habenula via the dorsal raphe nucleus.

SEM revealed that vehicle-treated rats had a cortico-subcortical network with very weak effective connections as illustrated by path coefficient values generally close to zero. In contrast, responders had strong negative path coefficients between the prelimbic cortex and the lateral habenula and dorsal raphe nucleus. Furthermore, the responders were clearly differentiated from the non-responders by the opposite influences between the dorsal raphe and the prelimbic cortex and lateral habenula. These regions showed positive path coefficients in the non-responders and negative path coefficients in the responders. This means that in responders for every unit increase in cytochrome oxidase activity in the dorsal raphe, there were corresponding decreases in the lateral habenula and prelimbic cortex. The opposite was true for the non-responders. The non-responders also had a high positive path coefficient from the interpeduncular nucleus to the dorsal raphe that was absent in responders and vehicle groups. Finally, rats treated with fluoxetine showed a strongly positive coefficient for the habenulointerpeduncular path, which was independent of treatment response.

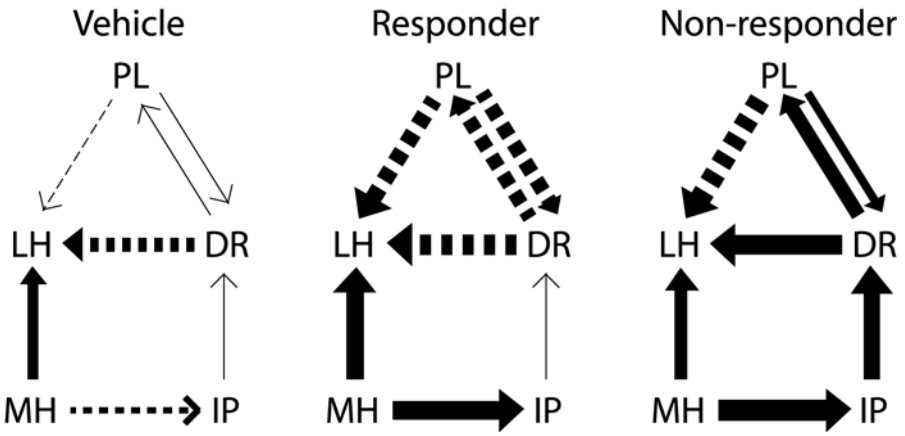

Figure 3.5. Structural equation model of cortico-subcortical interactions. The diagram illustrates the differences in the path coefficients (direct effects) between vehicle, responder and non-responders groups. Vehicle-treated subjects show weak connections in general. Responders show strong negative influences (dashed arrows) between the PL, DR and LH. Particularly, the strong negative reciprocal influences between the PL and DR differentiate responders from the vehicle and non-responders groups. Non-responders show mainly positive influences (solid arrows) between regions except between the PL and LH. They also show a strong positive influence arising from the IP to the DR that is absent in vehicle and responder groups. Animals that receive fluoxetine show a positive influence arising from the MH to the IP that is independent of treatment response. Abbreviations: PL (prelimbic), LH (lateral habenula), DR (dorsal raphe nucleus), MH (medial habenula), and IP (interpeduncular nucleus).

Table 3.7 Direct, indirect and total effects in structural equation model

VEHICLE GROUP

A. Total effects

	PL	LH	MH	IP	DR
PL	0.00		0.00	0.00	0.02
LH	-0.06		0.41	-0.01	-0.53
MH					
IP			-0.24		
DR	0.02		0.00	0.01	0.00

B. Direct effects

	PL	LH	MH	IP	DR
PL					0.02
LH	-0.05		0.41		-0.53
MH					
IP			-0.24		
DR	0.02			0.01	

C. Indirect effects

	PL	LH	MH	IP	DR
PL	0.00		0.00	0.00	0.00
LH	-0.01		0.00	-0.01	0.00
MH					
IP					
DR	0.00		0.00	0.00	0.00

RESPONDER GROUP

A. Total effects

	PL	LH	MH	IP	DR
PL	0.46		-0.01	-0.01	-0.80
LH	-0.27		0.66	-0.01	-0.62
MH					
IP			0.70		
DR	-0.84		0.02	0.03	0.46

B. Direct effects

	PL	LH	MH	IP	DR
PL					-0.55
LH	-0.63		0.66		-0.77
MH					
IP			0.70		
DR	-0.57			0.02	

C. Indirect effects

	PL	LH	MH	IP	DR
PL	0.46		-0.01	-0.01	-0.25
LH	0.36		-0.01	-0.01	0.15
MH					
IP					
DR	-0.26		0.02	0.01	0.46

NON-RESPONDER GROUP

A. Total effects

	PL	LH	MH	IP	DR
PL	0.20		0.31	0.40	0.65
LH	-0.67		0.66	0.23	0.38
MH					
IP			0.79		
DR	0.38		0.57	0.73	0.20

B. Direct effects

	PL	LH	MH	IP	DR
PL					0.54
LH	-0.79		0.48		0.74
MH					
IP			0.79		
DR	0.31			0.61	

C. Indirect effects

	PL	LH	MH	IP	DR
PL	0.20		0.31	0.40	0.11
LH	0.12		0.18	0.23	-0.36
MH					
IP					
DR	0.06		0.57	0.12	0.20

PL, prelimbic cortex. LH, lateral habenula. MH, medial habenula. IP, interpeduncular nucleus. DR, dorsal raphe nucleus.

3.4 DISCUSSION

The major effects of fluoxetine in this study are summarized as follows: 1) Fluoxetine exerted a protective effect against stress-induced depressive behavior among Holtzman rats. 2) The mean regional metabolism of the nucleus accumbens shell differentiated fluoxetine-treated from vehicle-treated subjects, but not responders from non-responders. 3) The infralimbic cortex and medial septum may play important roles in mediating antidepressant behavioral effects by contributing opposite influences as evidenced by their opposite neural activity correlations with FST immobility. 4) The cortico-cortical correlogram revealed complex interactions among responders that were less evident among non-responders and absent in the vehicle-treated group. 5) SEM analysis further revealed that direct path influences between the dorsal raphe nucleus and the prelimbic cortex and lateral habenula switched from negative to positive between fluoxetine-responders and non-responders, respectively. The differences in effective connectivity between the prelimbic cortex, lateral habenula and dorsal raphe may represent an important mechanism mediating response to antidepressants. Functional differences between responders and non-responders to fluoxetine treatment involved areas that have been previously associated with depression and anxiety; however, the interactions between these regions, and not mean changes in metabolic activity, provided greater insight into the brain mechanisms underlying treatment resistance. These results are particularly interesting in light of evidence showing that reciprocal interactions between dorsal cortical regions and ventral limbic structures are correlated with response to antidepressants among humans (Mayberg, 2003).

3.4.1 Effect of Fluoxetine on FST-Induced Immobility and Regional Brain Metabolism

Previous studies have repeatedly shown that treatment with standard antidepressant drugs including SSRIs can block FST-induced immobility in rodents

(Cryan *et al.*, 2005b; Detke *et al.*, 1997; Porsolt *et al.*, 1977). As a result, the FST is considered a useful screening tool for new antidepressant compounds (Porsolt *et al.*, 1977; Porsolt *et al.*, 1978). Unlike Detke et al. (1997), we did not find a significant effect of fluoxetine treatment on absolute immobility scores during the post-treatment FST, only a trend in this direction. However, inspection of the data revealed large individual differences which were present before treatment, which could have masked a treatment effect. Therefore, to control for this baseline variability, we calculated a measure of FST improvement for each subject by dividing its pre-treatment immobility by its post-treatment immobility. Thus, values less than 1 indicate a worsening of performance between tests (increase in immobility), and values greater than 1 indicate an improvement in performance (decrease in immobility). By this novel measure, fluoxetine significantly improved FST performance.

When examined within the context of the fluoxetine-treated group, these ratios also provide an index of the magnitude of treatment response for each subject. The treatment-response index for the fluoxetine group ranged from 0.71 to 2.02. Viewed in this light, it is clear that there are individual differences in treatment response among Holtzman rats, with some rats not responding or perhaps getting worse after fluoxetine treatment. However, it is unclear *a priori* what index value constitutes an adequate treatment response analogous to depression remission. Therefore, to establish whether the distribution of scores supports the separation of fluoxetine-treated animals into two or more qualitatively distinct groups, we performed a cluster analysis. Cluster analysis aims to sort subjects into an optimal number of clusters such that the degree of association within a cluster is maximal and the degree of association between clusters is minimal. This analysis identified the existence of two clusters, which we termed responders and non-responders. The treatment-response index of responders ranged from 1.35 to 2.02 with a mean of 1.63; non-responders ranged from 0.71 to 1.22 with a

mean of 1.02. By these criteria, 39% of subjects are responders and 61% are non-responders. Notably, all of the non-responders fall within the range of vehicle-treated controls (0.11 to 1.75 with a mean of 0.83), and only 2 vehicle controls would have been falsely classified as responders by these criteria. Or, viewed another way, there is a 13% incidence of spontaneous improvement in the FST absent any treatment intervention, and fluoxetine-treated animals are 3 times more likely than untreated animals to show significant FST improvement.

The only mean regional difference between fluoxetine and vehicle-treated animals was a decrease in the nucleus accumbens shell. The nucleus accumbens was also the region with the largest decrease in cytochrome oxidase activity in a study with another antidepressant, amitriptyline (Gonzalez-Pardo *et al.*, 2008). However, in the current study, further analysis between fluoxetine treatment responders, non-responders and vehicle-treated did not show any mean differences between groups. Hence the behavioral antidepressant response was unlikely mediated by the nucleus accumbens shell in isolation of a larger network. This indicates that the mean difference observed in the nucleus accumbens shell may be a nonspecific effect of fluoxetine unrelated to treatment response.

The negative correlation between forced swim immobility and metabolic activity in the infralimbic cortex among fluoxetine-treated subjects may be related to the inhibitory influence of the infralimbic cortex on fear-induced freezing behavior (Bruchey *et al.*, 2007). According to a structural equation model proposed for extinction of fear-induced freezing (immobility), rats that underwent fear extinction recruited the infralimbic cortex (Bruchey *et al.*, 2007). Specifically, rats that better extinguished fear-induced immobility had higher infralimbic activity correlated with lower immobility, as we found among fluoxetine-treated subjects in the FST. In addition, since vehicle-treated subjects lacked this relationship, better extinction of fear-induced immobility could potentially

underlie the fluoxetine-reduced immobility in the FST. In fact, animals that are susceptible to helplessness, such as the Holtzman strain (Padilla *et al.*, 2009; Wieland *et al.*, 1986), are considered models for depression and PTSD, two highly overlapping stress-related psychopathologies, the latter being linked to reduced extinction of fear memories (American Psychiatric Association, 2000a).

However, our findings in the infralimbic cortex contrast with findings from the human literature in which lower, rather than higher, activity in the subgenual cingulate (homologous to infralimbic cortex in rats) has been related to an antidepressant effect of fluoxetine (Mayberg *et al.*, 2000). The reason for this discrepancy is not clear. One possibility is that the negative correlation between forced swim immobility and infralimbic activity could be attributed to insufficient duration of fluoxetine therapy (two weeks in our study versus six weeks in humans). Even though behavioral changes were evident after two weeks, other compensatory changes in the brain may require more time. It has been reported that the therapeutic delay of 3-6 weeks before the effects of antidepressant medication and electro-convulsive treatment are seen may be explained by the time required for downstream changes in structural and functional synaptic plasticity to re-establish homeostasis in the neural network involved in depression (Pittenger & Duman, 2008).

Nonetheless, infralimbic activity was significantly correlated with immobility during the FST only among the fluoxetine treatment group. Post-mortem studies of depressed patients showed decreased serotonin transporter binding in the brainstem, subgenual and anterior cingulate, suggesting that these sites are likely to be the most affected by changes in serotonin concentrations which can lead to other adaptive changes (Arango *et al.*, 1995; Malison *et al.*, 1998; Mann *et al.*, 2000). The subgenual cingulate, or infralimbic cortex, is connected with cortical and subcortical structures implicated in the pathophysiology of depression including the anterior cingulate, dorsal-medial prefrontal

cortex, dorsal raphe nucleus and habenula (Goldapple *et al.*, 2004; Lozano *et al.*, 2008; Mayberg *et al.*, 1999; Vertes, 2004). In contrast to the infralimbic cortex, cytochrome oxidase activity in the medial septum was positively correlated with immobility among fluoxetine-treated animals. Intraseptal injections of a serotonin (5HT1A) agonist have produced antidepressant effects in forced swim and learned helplessness paradigms (Martin *et al.*, 1990; Schreiber & De Vry, 1993), whereas medial septal lesions reduce anxiety (Treit & Menard, 2000). Therefore, increased cytochrome oxidase activity of the medial septum could be related to increased anxiety among animals that do not respond to fluoxetine and show greater immobility or depressive-like behavior.

3.4.2 Fluoxetine-Responders and Non-responders Showed Differences in Functional Connectivity between Infralimbic and Other Frontal Cortical Regions

In general, only 55 to 70 percent of patients respond to antidepressant treatment (Keller, 2005; Nelson *et al.*, 2008). As such, approximately 30 to 45 percent of patients are resistant to antidepressants. As mentioned previously, 61% of our Holtzman rats were non-responders based on their FST treatment response ratio. The amount of non-responders was slightly higher than what has been reported with humans. This may be due to the fact that we used a stress-susceptible strain of rats (Padilla *et al.*, 2009; Spivey *et al.*, 2008) that may be less responsive to antidepressants. However, having used a susceptible population to examine the neural effects of antidepressants may increase the translational relevance of the results.

Limbic-cortical dysregulation has been proposed as a network model for depression and is characterized by dorsal cortical hypoactivity with hyperactive ventral limbic areas in humans (Mayberg, 1997). Increased metabolic activity in dorsolateral prefrontal cortex correlated with reduction in ventral limbic regions, including the subgenual cingulate, is associated with an antidepressant response after 6 weeks of SSRI treatment among depressed patients (Goldapple *et al.*, 2004; Mayberg *et al.*,

2000). Our results were in agreement with this network model based on the cortico-cortical differences observed between vehicle, responder and non-responder groups. The correlations between the infralimbic cortex with dorsolateral and insular cortical regions switched from strongly positive to highly negative between vehicle and responder groups, respectively, and showed functional decoupling among non-responders. The infralimbic cortex is often a therapeutic target for treatment-resistant depression. In previous human and rodent studies, lesions or electrical stimulation of this area (which is thought to lead to suppression of neural activity) have produced antidepressant effects (Cosgrove & Rauch, 1995; Lozano *et al.*, 2008; Hamani *et al.*, 2010). A dissociation of this ventral limbic area from dorsal cortical structures may represent a unique mechanism for treatment resistance. Not only was the infralimbic cortex not correlated with other prefrontal structures among non-responders, but there was much less functional connectivity in general between prefrontal regions including the prelimbic, dorsal and lateral frontal, insular and orbital cortices. The cortico-cortical correlogram provided a way of illustrating the complexity of interactions that occurred among fluoxetine responders, in comparison to the other groups. Vehicle–treated animals lacked any complexity in their regional interactions, and showed only positive correlations among superficial cortical layers. Meanwhile the non-responders appeared to be intermediate between the vehicle and the responder groups, failing to show the same pattern of functional coupling as seen with the responders.

However, the question remains as to why we did not observe mean differences in these regions as predicted by human studies. One possible explanation is that our sample size is somewhat small for the responder group (n = 7) in a rat population that shows large individual differences in both brain metabolism and behavior. Such high variability inherently favors the detection of correlative relationships and disfavors the detection of group mean differences. Two regions can also show changes in covariance

without a significant change in regional activity (McIntosh & Gonzalez-Lima, 1994) and it has been suggested that behavioral-related plasticity first involves a change in covariances between neural elements, which then leads to a change in regional activity (Ahissar *et al.*, 1992). Furthermore, since single brain regions do not operate in isolation of each other, increased differentiation of the interactions in the prefrontal network may be more representative of the mechanism underlying a therapeutic response.

3.4.3 Limbic-Subcortical Pathways Involving the Dorsal Raphe and Habenula Represent an Important Network for Antidepressant Action

Changes in metabolic activity of the ventral limbic cortex have been linked to an antidepressant response (Mayberg, 1997; Mayberg, 2003). However, less is known about the interactions that occur between the ventral limbic regions and subcortical structures that can potentially explain treatment response or resistance. Interestingly, both the habenula and dorsal raphe have been linked to human depression and hypersensitivity to stress in animals (Grahn *et al.*, 1999; Hikosaka *et al.*, 2008; Shumake & Gonzalez-Lima, 2003; Morris *et al.*, 1999). Increased activity in the habenula is related to depressive-like behavior in numerous studies (Caldecott-Hazard *et al.*, 1988; Morris *et al.*, 1999; Rosier *et al.*, 2009; Shumake *et al.*, 2003). Specifically, patients experiencing transient depressive episodes elicited by acute tryptophan depletion showed increased blood flow or metabolism in the habenula that correlate with ratings of depressed mood and inversely with plasma tryptophan levels (Morris *et al.*, 1999; Rosier *et al.*, 2009). Furthermore, animal studies have implicated the habenula in many processes affected in depression, such as the sleep-wake cycle (Haun *et al.*, 1992; Valjakka *et al.*, 1998), reward mechanisms (Boyd & Celso, 1970; Gallistel *et al.*, 1985; Sutherland & Nakajima, 1981), antinociception (Benabid & Jeaugey, 1989; Fuchs & Cox, 1993), behavioral inhibition (Lee & Huang, 1988) and hormonal responses to stress (Sandyk, 1991; Sutherland, 1982). The medial and lateral habenula receive input from the limbic system

(e.g. prelimbic cortex, septum, hypothalamus, preoptic area, nucleus of the diagonal band) and the lateral habenula projects to regions such as the ventral tegmental area and dorsal raphe (Hikosaka *et al.*, 2008; Vertes, 2004). This positions the habenula in a critical role as mediator of the interactions that occur between limbic regions and other nuclei that have previously been linked to depression and anxiety. For example, serotonin (5HT) neurons within the dorsal raphe are normally under autoinhibitory control of 5HT1A somatodendritic receptors (Cooper *et al.*, 2003). The 5HT1A autoreceptors are especially susceptible to desensitization produced by high levels of extracellular 5HT which occur after exposure to inescapable stress (Maier & Watkins, 2005). Desensitization of 5HT1A autoreceptors and increased 5HT release from the dorsal raphe appear necessary for the development of learned helplessness (Grahn *et al.*, 1999; Maier *et al.*, 1993; Petty *et al.*, 1994), and the habenula plays an important role in mediating increased dorsal raphe activity in response to inescapable stress (Amat *et al.*, 2001). In fact, lesioning the habenula can prevent the rise in serotonin release from the dorsal raphe following inescapable shock and blocks the development of learned helplessness (Amat *et al.*, 2001).

The habenula showed many functional connectivity differences between groups involving the dorsal raphe, interpeduncular nucleus, prelimbic, lateral frontal, and insular cortices, and periaqueductal gray. The prelimbic, dorsal frontal and lateral orbital cortices also showed strong negative correlations with the periaqueductal gray among responders as opposed to a general decoupling observed in the vehicle and non-responders groups. The periaqueductal gray is implicated in the regulation of fight or flight, and panic-like autonomic and behavioral responses (Bandler *et al.*, 2000). Differences in functional connectivity in these paths may represent important mechanisms underlying stress-coping strategies and response to antidepressants. The deep lateral frontal cortex was also positively correlated to the ventral tegmental area

among responders while the opposite pattern was observed in non-responder and vehicle groups. Increased activity in the dorsolateral prefrontal cortex and ventral tegmental area have been related to antidepressant effects (Friedman *et al.*, 2009; Mayberg *et al.*, 2000). The ventral tegmental area plays an important role in reward and motivation thus improved functional connectivity between these cortical and subcortical nuclei may represent another important treatment effect. Structural equation modeling was applied in order to better understand the complexity of these network interactions. Each path coefficient in a structural equation model represents an unknown variable, and each inter-regional correlation is a known variable. To avoid unsolvable models we needed to have less unknown than known variables, therefore regions such as the periaqueductal gray, ventral tegmental area, lateral frontal and orbital cortex were excluded from the model for simplification purposes and regions with known anatomical interconnectivity and significantly different correlations between groups such as the habenula, interpeduncular nucleus, dorsal raphe, and prelimbic cortex were included.

As seen in the final model, the dorsal raphe exerted direct negative influences on the lateral habenula and prelimbic cortex in the responder group, and positive influences in the non-responder group. Increased metabolism in the lateral habenula and reduced brain serotonin levels have been reported in animal models of depression and these effects can be reversed by antidepressant drugs (Caldecott-Hazard *et al.*, 1988; Keck *et al.*, 2005; Umriukhin *et al.*, 2002). It is possible that increased levels of serotonin, due to reuptake inhibition, leads to reduced habenula metabolism. This could be a potentially important mechanism of antidepressant action since decreased habenula metabolic activity is related to reduced depression symptom severity among humans (Morris *et al.*, 1999) and habenula ablation completely blocks the development of learned helplessness in rats (Amat *et al.*, 2001). Therefore, it is possible that negative influences

of the dorsal raphe on the lateral habenula and prelimbic cortex may represent important pathways underlying response to SSRI antidepressants.

Another network model difference that distinguished non-responders from the other two groups was the strong positive influence from the interpeduncular nucleus to the dorsal raphe. The interpeduncular nucleus receives more acetylcholine input than any other region in the mammalian brain (Woolf & Butcher, 1985) and there is evidence that excessive cholinergic activity is implicated in the etiology of depression (Charles *et al.*, 1994; Dilsaver & Coffman, 1989; Janowsky *et al.*, 1983; Steingard *et al.*, 2000). The interpeduncular nucleus, which receives a major projection from the medial habenula (Contestabile & Flumerfelt, 1981) was metabolically hyperactive in congenitally helpless rats and may contribute to helplessness susceptibility (Shumake & Gonzalez-Lima, 2003). Therefore, a strong effective connectivity between the interpeduncular nucleus and subcortical nuclei such as the dorsal raphe could be a potential mechanism underlying antidepressant treatment resistance. An advantage of comparing fluoxetine responders, non-responders and vehicle-treated subjects was that we could identify nonspecific effects of fluoxetine that were unrelated to treatment response, such as the common fluoxetine positive influence seen in the medial habenula-interpeduncular pathway among responders and non-responders. These differences in effective connectivity between treatment responders and non-responders provided a novel insight into the changes that might occur in an adaptive network resulting in a behavioral treatment response. Lack of these changes could identify treatment-resistant individuals that require additional or different pharmacologic or non-pharmacologic interventions. Future studies may examine the network effects of other treatments (noradrenergic drugs, infralimbic brain stimulation, etc.) in subjects that do not respond to SSRIs. Further investigation into the network effects of diverse antidepressant treatment

modalities may lead to the development of evidenced-based treatment plans that are specific to the needs of individual patients.

In conclusion, several studies have reported that mean changes in regional brain metabolic activity are correlated with an antidepressant treatment response, but few studies have examined the effects on widespread cortical-subcortical functional connectivity and specific data-driven networks. Human neuroimaging studies have identified reciprocal changes in dorsal cortical and ventral limbic areas that are correlated with a treatment response; however, it is difficult to obtain subcortical resolution with this approach. Psychopathologies such as depression and anxiety are more likely to be mediated by changes in networks involving both cortical and subcortical regions. Identification of these networks might provide more information on how to empirically determine treatment response. This study is unique because it characterized the neural networks underlying treatment response involving both cortical and subcortical regions among rodents. In addition, this study represents the first application of structural equation modeling to identify effective connectivity differences between fluoxetine responders and non-responders using an animal model of depressive behavior. Understanding the mechanisms that underlie treatment resistance can influence treatment decisions and improve antidepressant therapy guidelines.

Chapter 4 Identification of stress-susceptible and resistant subjects within the Holtzman strain: behavioral and physiological characterization

Learned helplessness in animals has been used to model disorders such as depression and post-traumatic stress disorder (PTSD), but there is a lack of knowledge concerning which individual behavioral characteristics at baseline can predict helpless behavior after exposure to inescapable stress. The first aim of this study was to determine behavioral predictors of helplessness using the novel and familiar open-field test, sucrose consumption and passive harm avoidance tasks before learned helplessness training and testing. Individual differences in physiologic responses to restraint stress were also assessed as potential predictors. A cluster analysis of escape latencies from helplessness testing supported the division of the population into approximately 50% helpless and 50% non-helpless. Linear regression analyses further revealed that increased novelty-specific activity predicts susceptibility to learned helplessness ($r > 0.45$), as defined by increased latency to escape shock. There were no mean differences in heart rate, heart rate variability, and plasma corticosterone between helpless and non-helpless rats; however, a lower heart rate during the last 15 sec of restraint stress predicted higher escape latencies. Our most important finding was that by using an innocuous screening tool such as the novel and familiar open-field test, it was possible to identify subjects that were susceptible to learned helplessness. Individual differences in bio-behavioral responses to stress could be used to identify at-risk subjects before the development of the helpless phenotype.

4.1 INTRODUCTION

Current evidence supports the relationship between individual personality traits and the predisposition to neuropsychiatric disorders such as depression and post-traumatic stress disorder (PTSD) (Cloninger *et al.*, 2006; Enns & Cox, 1997; Oquendo *et*

al., 2004; Richman & Frueh, 1997). In fact, animals have been selectively bred and used as disease models to characterize behavioral correlates of these conditions (Pucilowski *et al.*, 1993; Vollmayr *et al.*, 2004). One such model is the congenitally helpless rat, which was selectively bred for a genetic predisposition to helpless behavior as defined by the learned helplessness paradigm (failure to escape shock after a previous exposure to inescapable shock) (Henn & Edwards, 1994). Behavioral characteristics of congenitally helpless rats include increased novelty-induced activity, reduced reward sensitivity, and an impaired ability to extinguish conditioned fear (Shumake *et al.*, 2005; Vollmayr *et al.*, 2004). Novelty-induced activity is characterized by increased exploratory activity in a novel environment (Ballaz *et al.*, 2007; Stead *et al.*, 2006). Intuitively, increased exploration in a new environment seems to contradict susceptibility to helplessness. However, it is consistent with a predisposition to PTSD, given the association of this disorder with increased novelty seeking, as measured by the Cloninger Tridimensional Personality Questionnaire (Richman & Frueh, 1997; Wang *et al.*, 1997).

Based on data from the congenitally helpless strain, it was hypothesized that increased exploration in response to novelty, reduced consumption of a rewarding sucrose solution and enhanced passive avoidance of a harmful stimulus would predict helpless behavior (defined by high escape latencies) in a randomly-bred population of rats. To date, no studies have examined the predictive value of all three behavioral dimensions in the development of helplessness. Therefore the first aim of this study was to evaluate potential behaviors that predict a helpless phenotype using the open-field, sucrose consumption and passive avoidance tasks before exposure to inescapable shock.

Individual differences in the responsiveness of the stress pathways including the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis have also

been studied in animals and humans (Jemerin & Boyce, 1990; Levine, 1975; Marchei *et al.*, 2009; Pariante, 2009; Puttonen *et al.*, 2008; Sheps & Sheffield, 2001). For instance, infants who displayed greater overt anxiety during maternal separation and exposure to novelty had significantly higher urinary cortisol excretion during the episodes (Tennes *et al.*, 1977) and adult rats that were not handled as neonates by experimenters had less effective negative feedback regulation of adrenocorticotropic hormone (ACTH), resulting in greater corticosterone secretion during stress (Meaney *et al.*, 1988). Furthermore, increased levels of glucocorticoid hormones - the main product of the HPA axis - has been shown in a significant percentage of depressed patients (Pariante, 2009). However, a dissociated HPA axis was observed in congenitally helpless rats, characterized by a hyperactive paraventricular hypothalamus (Shumake *et al.*, 2001) and reduced plasma corticosterone both at baseline (Edwards *et al.*, 1999) and in response to stress (Edwards *et al.*, 2000; King & Edwards, 1999).

Also, reduced heart rate variability has been observed in depressed patients when compared with non-depressed controls, and the risk of subsequent cardiac events for patients with heart disease has been shown in numerous studies to be related to decreased heart rate variability and/or increased sympathetic tone (Carney *et al.*, 1988; Carney *et al.*, 1993; Frasure-Smith *et al.*, 1995; Sheps & Sheffield, 2001). Furthermore, the presence of major depressive disorder was the best single predictor for the occurrence of cardiac events, and the predictive value was independent of the extent of coronary disease, smoking status, and left ventricular ejection fraction (Carney *et al.*, 1988).

Temperament or personality is also linked to physiologic parameters such as heart rate and cortisol. For example, impulsivity, a personality factor that correlates with novelty seeking (Zuckerman & Cloninger, 1996) has been associated with lower levels of resting heart rate (Mathias & Stanford, 2003), and novelty seeking has been inversely

correlated with resting stress hormone concentrations (i.e. cortisol) (Tyrka *et al.*, 2007). Furthermore, among PTSD combat veterans, increased novelty seeking was inversely correlated with cortisol levels (Wang *et al.*, 1997). The second aim of this study was to identify individual differences in the physiological response to restraint stress, including heart rate, heart rate variability and plasma corticosterone that could predict helpless behavior among Holtzman rats. The hypothesis was that animals with increased susceptibility to helplessness would show higher novelty-specific activity that would correlate with reduced plasma corticosterone and heart rate measures.

4.2 MATERIALS AND METHODS

4.2.1 Subjects

Subjects were 21 male Holtzman rats obtained from Harlan (Madison, WI) at postnatal day 30 (PD 30). Animals were housed 2–3 per cage and maintained on a 12 h/12 h light/dark photoperiod in a facility accredited by the Association for the Assessment of Laboratory Animal Care International. Food and water were available *ad libitum*. Subjects were handled and weighed for 5 minutes every day throughout 9 consecutive days prior to starting behavioral experiments. All experiments occurred during the light phase between 0700 h and 1900 h. Experiments were done in accordance with NIH guidelines for the use of experimental animals and were approved by the University of Texas Institutional Animal Care and Use Committee.

4.2.2 Apparati

The open field chamber (43.2 cm^2) consisted of clear plastic sides 30.5 cm high and a white plexiglass floor. Activity was detected by arrays of infrared light beam motion detectors (16 x 16, 2.5 cm apart) at the sides of each chamber, thus creating a detection grid. Two arrays of detectors were located 1 cm above the floor, and another array was

located 13 cm above the floor, to detect rearing. The chambers were controlled by the Activity Monitor program, version 5.10 (Med Associates, St. Albans, VT).

The sucrose chambers consisted of two 30 cm × 25 cm × 20 cm operant chambers (Med Associates, St. Albans, VT). The bottles were designed by Med Associates for use in these operant chambers, and a light beam passing in front of the tip of the bottle registered the time the rat spent drinking. The bottles were located on the right side of the chamber.

The step-down apparatus for passive avoidance testing consisted of one 30 cm × 25 cm × 20 cm operant chamber (Med Associates, St. Albans, VT). Shocks were delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). An acrylic platform (23.4 cm × 14.3 cm × 2.2 cm) was placed on the grid floor.

Two inescapable shock chambers (30 cm x 25 cm x 20 cm) (Med Associates, St. Albans, VT) were enclosed in sound-attenuated boxes and illuminated by a red light. The apparatus had two sides of aluminum, with clear plexiglass for the front, back, and top. A soapy solution was placed in the tray beneath the chambers to provide a distinct olfactory cue for the inescapable context. Shocks were delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). The chamber was controlled by MED-PC, version 4 (Med Associates), using a program written in the MEDSTATE language.

The shuttle box (42 cm x 16 cm x 25 cm) consisted of two compartments of equal size, separated by a door (11 cm x 9 cm) that remained open throughout the session. The chamber was enclosed in a sound-attenuated box and illuminated by a white light. Two sides of the chamber were aluminum, with clear plexiglass for the front, back, and top. Shocks were delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). The subject's

position was detected by eight sets of infrared light beam motion detectors, located 2 cm above the grid floor, spaced 4.4 cm apart from each other, on both sides of the chamber. The chamber was controlled by MED-PC, version 4, using a program written in the MEDSTATE language. This program used beam breaks of the two pairs of beams located at either end of both sides of the chamber as the contingency for terminating shock, to score a complete crossing. An iodine solution was placed in the tray beneath the chamber to provide a distinct olfactory cue.

Heart rate measurements were performed using a Mouse Ox™ oximeter (Starr Life Sciences Corp, Oakmont, PA). Pulse rates were measured via cyclic changes in absorption of light energy from red and infrared LEDs (light emitting diodes) as it passed through the tissue. These cyclic changes in light absorption are due to the presence of changing quantities of blood that occurred with every heartbeat. Animals were placed inside restraint tubes (20.2 cm x 5.08 cm) for 2 to 5 minutes.

4.2.3 Behavioral experiments

Behavioral experiments took place during six consecutive days (Table 4.1). All animals were tested for open field (OF) activity during the first day of experiments (novel OF) on PD 40 to determine if behavioral characteristics predictive of helplessness were present before learned helplessness training. Each animal was placed in the same corner of the open field chamber and behavior was recorded for 10 minutes. The chambers were washed with a diluted Bio-clean solution between each session.

Measures included ambulation time (seconds spent breaking horizontal beams), rearing counts (vertical beam breaks), short movement counts (movement without displacement), average velocity of ambulation (cm/sec), thigmotaxis (seconds spent in the 62 % periphery versus 38 % center of the open field as a measure of risk-taking or anxiety-like behavior) and resting time (seconds spent without breaking beams). Measures were automatically scored by a computer using MED-PC software. Subjects

were re-tested in the open field (familiar OF) the following day. This was done in order to assess whether the behavior was reflective of exploratory activity specific to a novel environment.

Immediately following the familiar open-field session, baseline consumption of a 5% sucrose solution or water was assessed for 30 minutes where rats had free access to two bottles containing the sucrose solution or drinking water. The sucrose test was repeated on the following day and the bottles were counterbalanced with respect to their position the day before. This was performed to account for place preference of the bottles (front or back). A sucrose-to-water drinking ratio was computed for both the baseline and second-day sessions. This ratio is meant to reflect the preference for sucrose solutions over water as an indicator of reward-seeking behavior.

A modified step-down passive avoidance task was performed based on previously described protocols (Cammarota *et al.*, 2005; Henningsen *et al.*, 2009). The procedure consisted of two trials: an acquisition trial and a retention trial with an inter-trial interval of 24 h. *Acquisition trial*: Immediately after the second sucrose test session, animals were placed on the acrylic platform inside the passive avoidance chamber. As soon as the rat stepped down onto the grid floor with all four paws, a 0.3 mA shock was delivered continuously for a maximum of 180 s or a total number of three step downs. Rats could avoid the shock by remaining on the acrylic platform. A maximum of three step downs was used as a criterion to control for the amount of shock the animals received. *Retention trial:* Rats were exposed to the same context with exception of the footshock. They were placed on the acrylic platform and the latency to step down onto the un-electrified grid floor with all four paws was determined using a maximum duration of 180 s. Only step-down latencies were reported and latencies above 180 s were counted as 180 s.

Immediately following the retention trial, subjects were trained in the inescapable shock chamber to induce the helpless phenotype (Hunziker & Dos Santos, 2007; Maier *et al.*, 1973; Overmier & Seligman, 1967). Each session included 60 trials of 10 s duration 0.7 mA shocks. Pseudorandom inter-trial intervals consisted of durations ranging from 10 to 110 seconds.

On the fifth day, subjects were tested with the escapable shock paradigm using a shuttle box to measure escape behavior. First, subjects were tested with fifteen trials of a fixed ratio (FR) 1 schedule consisting of crossing from one side of the box to the other to terminate the shock. The maximum footshock duration was 15 seconds, after which the shock was terminated if the subject had not escaped. This was followed by 15 trials of a FR2 schedule in which animals had to cross twice; in other words, rats had to return to the compartment where the shock was initiated in order to escape the shock. During the FR2 trials, the maximum footshock duration was 30 seconds. There was a 1-minute interval between FR1 and FR2 escape tasks. Based on our previous results using Holtzman rats, a FR1 schedule is sufficient to observe a learned helplessness effect (Padilla *et al.*, 2009), therefore we included an equal number of trials for both types of escape responses to obtain comparable results with both schedules. Latency to escape was automatically scored using MED-PC software.

On the last day, subjects were placed inside the restraint tube for heart rate measurements. To increase pulse distention and obtain a stable LED signal, the restraint tube was placed on top of a heating pad. The oximeter sensor was placed on the rat's tail using a specialized clip (Starr Life Sciences, Oakmont, PA). Animals were covered using a dark cloth to avoid external light from interfering with the LED signal. After 1 - 4 minutes of stabilization time, the heart rate was recorded for 1 minute; therefore the total restraint time was 2-5 minutes. Average heart rates were reported as four 15-second bins as well as overall mean heart rate. Heart rate variability, a

parameter for autonomic control of the heart (Friedman & Thayer, 1998), was determined using the mean standard deviation of measurements taken throughout the 1 minute period (Pattij *et al.*, 2002; Vinkers *et al.*, 2009). Immediately following the heart rate measurement, animals were decapitated. Trunk blood was collected in BD vacutainer® heparinized 3 ml tubes (BD, Franklin Lakes, NJ) for assessment of plasma corticosterone.

4.2.4 Corticosterone assay

Plasma was separated by centrifuging for 10 minutes at 4°C with a speed of 2500 rpm. Corticosterone was detected in 100 µl samples using the Correlate-EIA™ enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI), following the procedure recommended by the manufacturer.

Table 4.1 Experimental Design

Experiment	Postnatal day (PD)
Handling	31-39
Novel open field	40
Familiar open field	41
Sucrose test I (baseline)	41
Sucrose test II	42
Passive avoidance I (step-downs)	42
Passive avoidance II (no shock)	43
Learned helplessness training	43
Learned helplessness testing	44
Heart rate measurement	46
Trunk blood collection (corticosterone assay)	46

4.2.5 Statistical Analyses

Two-step clustering analysis was applied to identify how subjects' escape behavior can differentiate them into distinct groups or clusters (Chourbaji *et al.*, 2005; Espejo & Mir, 1993). This type of analysis consisted of calculating the dissimilarity or the distance between individuals based on their latencies to escape shock. Afterwards, a one-way analysis of variance (ANOVA) of open-field activity, latency to step down, corticosterone levels, heart rate average and heart rate standard deviation was performed to determine significant differences between separate groups. Sucrose consumption (2 x 2, Group x Session) and heart rate measurements (2 x 4, Group x Bins) were analyzed with repeated measures ANOVA and simple effects tests as needed.

Linear regression analyses were used to determine whether a predisposition for helpless susceptibility might be reflected in the open-field activity, sucrose consumption, passive avoidance and physiologic parameters. Linear regressions were analyzed between the average escape latencies of each subject in the FR1 and FR2 learned helplessness paradigm and those subjects' open field, sucrose test, step-down latency, heart rate and plasma corticosterone measurements. Open-field activity variables were analyzed using both sums (novel plus familiar) and ratios (novel to familiar) of the two open-field sessions. The former served as an index of general activity, while the latter reflected the proportion of activity that was novelty-specific. Standardized beta coefficients (β) and variance (r^2) were calculated for each regression.

A stepwise discriminant analysis of novelty-specific open-field variables was performed to determine which variable or combination of variables best discriminates helpless from non-helpless subjects. A separate discriminate analysis was performed on physiologic parameters. Stepwise selection of variables to enter into the equation was based on minimizing Wilks' lambda (Λ).

Pearson product-moment correlations between open field behavioral measures and physiologic parameters were calculated for all subjects. A jackknife procedure was performed in which each individual subject was dropped from a group, and then correlations were calculated again without that subject's data. This procedure was iterated until each subject had been sequentially dropped and the analysis repeated. To minimize Type I error, correlations that remained significantly different from zero at $p < 0.05$ through all iterations were considered statistically reliable for further analysis.

A factor analysis was performed on novelty-specific indices to determine one principal component score of novelty-specific open-field behavior. Statistical significance for all analyses was set at $p < 0.05$ and data were analyzed with SPSS 11.5 and R 2.9.0 software.

4.3 RESULTS

4.3.1 Two step-cluster analysis of shuttle box escape behavior

A cluster analysis based on FR1 and a separate analysis using FR2 escape responses divided the same subjects into two distinct clusters with high and low escape latencies. These were termed the helpless and non-helpless groups (Table 4.2). The helpless group showed three times longer escape latencies as compared to the non-helpless group ($p < 0.01$).

Table 4.2 Cluster Analysis of Shuttle Box Escape Responses

Escape response (latency, sec)	Helpless (N = 10) mean ± SE	Non-helpless (N = 11) mean ± SE	Total sample (N = 21) mean ± SE
FR1	11.89 ± 0.62 *	3.53 ± 0.46	7.51 ± 1.00
FR2	28.13 ± 0.82 *	8.54 ± 1.30	17.87 ± 2.32

* $p < 0.01$, helpless vs. non-helpless

4.3.2 Means analysis of behavioral measures

Helpless animals had increased ambulatory time in the novel but not familiar open field session (Figure 4.1). To further illustrate differences between novelty-specific behavioral activation and general activity, novelty-specific and general activity indices were computed for several open field activity measures and are shown in Table 4.3.

Helpless subjects displayed greater levels of novelty-induced open-field activity compared to non-helpless Holtzman rats (Table 4.3). There were no differences between helpless and non-helpless subjects in general activity measures.

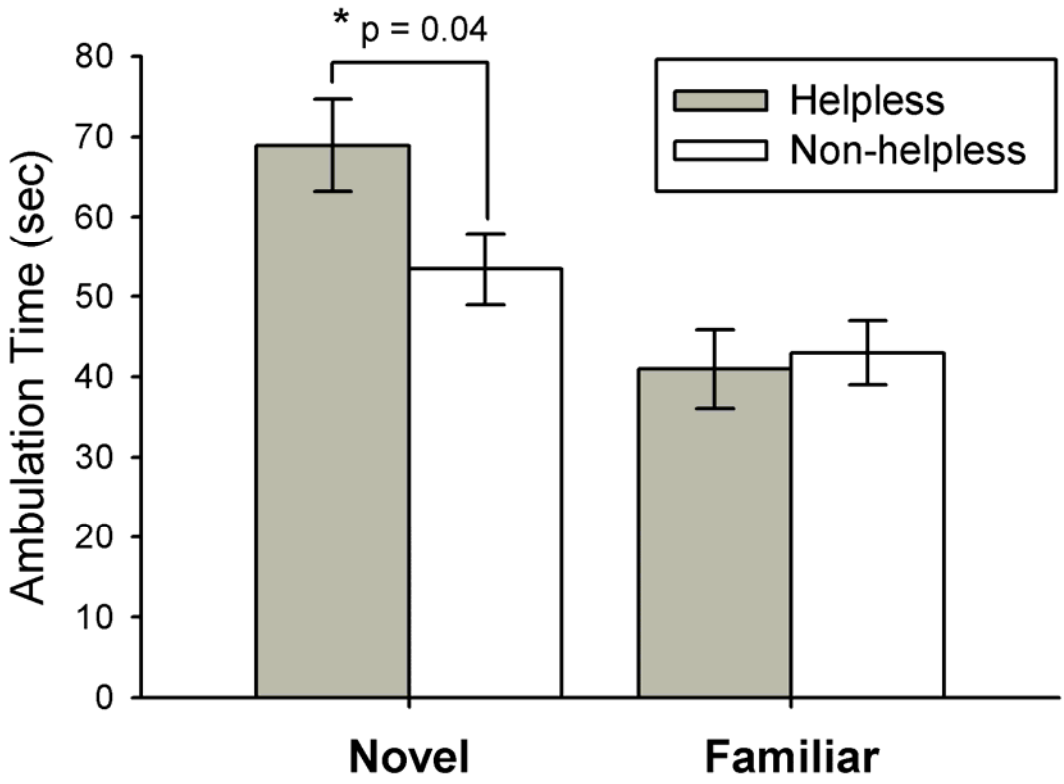

Figure 4.1 Helpless subjects showed increased time spent in ambulation in the novel but not familiar open field.

Table 4.3 Means of open-field measurements

Open-Field Activity Parameter	Helpless mean ± SE	Nonhelpless mean ± SE
Novelty-Specific Index (Ratio of Novel:Familiar sessions)		
Ambulation time	1.96 ± 0.29 *	1.31 ± 0.13
Short movement time	1.44 ± 0.08 **	1.18 ± 0.05
Vertical time	2.00 ± 0.40	1.31 ± 0.14
Average velocity	1.32 ± 0.06 **	1.10 ± 0.05
Center time	3.57 ± 1.02	1.55 ± 0.26
Resting time	0.86 ± 0.02 **	0.95 ± 0.02
General Activity (Sum of novel and Familiar Sessions)		
Ambulation time (sec)	110 ± 9.55	96.4 ± 7.35
Short movement time (sec)	250.6 ± 12.8	231.7 ± 13.5
Vertical time (sec)	148 ± 14.4	150.2 ± 18.8
Average velocity (cm/sec)	2.04 ± 0.08	2.03 ± 0.18
Center time (sec)	124 ± 18.4	109 ± 13.5
Resting time (sec)	811 ± 26.1	848 ± 22.3

* $p < 0.05$, ** $p < 0.01$

A 2 x 2 (Group x Session) repeated measures ANOVA showed no group difference on total seconds spent drinking sucrose, $F(1,19) = 0.55$, $p = 0.4$ or sucrose to water preference, $F(1,19) = 2.64$, $p = 0.12$. However, there was a significant group difference in time spent drinking water, $F(1,19) = 4.50$, $p < 0.05$, in which helpless subjects spent more time drinking water in both sessions versus non-helpless animals. Using water drinking time as a covariate in the analysis did not affect the results ($p > 0.5$). Bottle placement in the front or back of the chamber did not affect time spent drinking sucrose, water or sucrose preference ($p > 0.1$). There were no session by group interactions. In addition, there was no mean difference in step-down latencies between groups (Table 4.4).

Table 4.4 Means of sucrose consumption test and passive avoidance latency

Behavioral measure	Helpless (n= 10) mean ± SEM	Non-helpless (n=11) mean ± SEM	p value
Sucrose 1 (sec)	231.72 ± 38.90	275.87 ± 37.09	0.42
Water 1 (sec)	50.80 ± 7.65	40.16 ± 7.29	0.33
Sucrose/water 1	5.70 ± 1.29	7.73 ± 1.23	0.27
Sucrose 2 (sec)	290.24 ± 54.06	327.89 ± 51.54	0.62
Water 2 (sec)	47.66 ± 5.95	33.26 ± 5.67	0.10
Sucrose/water 2	7.50 ± 2.58	12.70 ± 2.46	0.16
Latency to step down (sec)	65.30 ± 18.58	95.09 ± 16.20	0.24

4.3.3 Linear regression analyses of behavioral measures and escape response

Novelty-specific activity predicted an escape response but not sucrose consumption or passive avoidance (Table 4.5). Novelty-specific open field activity parameters (short movement time and average ambulatory velocity) were significantly predictive of susceptibility to helplessness as evidenced by the high positive beta coefficients ranging from 0.46 to 0.51. In other words, the more behavioral activation that was specific to the novel open field, the higher the probability of showing increased escape latencies in the shuttle box test (Table 4.5). Multiple linear regression analyses indicated that novelty-specific ambulation, short movements, rearing, average velocity, and center time collectively explained 39% of the variance in FR1 escape latencies and 32% of the variance in FR2 latencies. However, general activity measures were not predictive of helpless behavior as evidenced by low standardized beta coefficients ranging from -0.23 to 0.26. Furthermore, general activity only accounts for 19% of the variance in FR1 and 13% of the variance in FR2 escape latencies ($p > 0.6$). Also, sucrose consumption was not predictive of escape behavior, with low standardized beta coefficients ranging from -0.11 to -0.27. Similarly, step-down latency did not predict helplessness (FR1, $\beta = -0.11$ and FR2, $\beta = -0.25$).

Table 4.5 Standardized beta coefficients for open-field variables predicting shuttle box escape responses

Open-Field Activity Parameter	FR1	FR2
Novelty-Specific Index (Ratio of Novel:Familiar sessions)		
Ambulation time	0.41	0.40
Short movement time	**0.51 ***	**0.50 ***
Rearing time	0.40	0.37
Average velocity	**0.48 ***	**0.46 ***
Center time	0.35	0.35
Resting time	**-0.52 ***	**-0.56 ***
General Activity (Sum of novel and Familiar Sessions)		
Ambulation time (sec)	0.18	0.23
Short movement time (sec)	0.15	0.26
Rearing time (sec)	-0.13	0.07
Average velocity (cm/sec)	-0.15	0.04
Center time (sec)	0.16	0.26
Resting time (sec)	-0.17	-0.23

* $p < 0.05$

4.3.4 Stepwise discriminant analysis of novelty-specific measures

Novelty-specific activity was the only behavioral measure that predicted helplessness. A stepwise discriminant analysis of novelty-specific open field indices (ambulation time, short movement time, rearing time, average velocity, and center time) was performed to determine which variable or combination of variables best discriminates helpless from non-helpless subjects based on covariance differences. The final discriminant function yielded one variable, ratio of average velocity, which classified 16 out of 21 subjects correctly ($\chi^2(1) = 8.5$, $p < .01$). This indicates that novelty-specific average velocity was the best predictor of helplessness susceptibility and that inclusion of additional variables did not improve predictability. Essentially, Holtzman rats with a novelty-specific average velocity ratio greater than 1.2 were 3 times more likely to develop helplessness than Holtzman rats with a ratio less than 1.2.

4.3.5 Means analysis of physiologic parameters

There were no significant mean differences in corticosterone levels, heart rate average, and heart rate variability between helpless and non-helpless Holtzman rats that were subjected to restraint stress (Table 4.6). Heart rate measurements were divided into four 15-sec bins. A 2 x 4 repeated measures ANOVA (Group x Bin) did not show group differences, $F(1,19) = 1.68$, $p = 0.21$. Simple effects analysis showed a trend for helpless subjects having a lower heart rate during the last 15-second bin ($p = 0.08$). There were no group interactions with the physiologic measures and using restraint time as a covariate did not affect the results for heart rate or plasma corticosterone.

Table 4.6 Means of physiologic parameters between helpless and non-helpless Holtzman rats

Physiologic parameter	Helpless (n= 10) mean ± SEM	Non-helpless (n=11) mean ± SEM	p value
Corticosterone	3234 ± 291.4	3023 ± 235.8	0.58
Heart rate average	455.81 ± 16.34	482.15 ± 12.45	0.21
Heart rate STDEV	12.83 ± 1.91	15.64 ± 2.25	0.36
Heart rate bin 1	460.17 ± 15.32	483.94 ± 14.61	0.28
Heart rate bin 2	456.05 ± 15.22	479.95 ± 14.51	0.27
Heart rate bin 3	457.46 ± 14.21	475.98 ± 13.55	0.36
Heart rate bin 4	449.53 ± 15.47	488.74 ± 14.75	0.08

4.3.6 Linear regressions analysis of physiologic measures and escape response

Linear regressions analysis between physiologic parameters and shuttle box escape latencies showed that a lower heart rate during the last fifteen seconds of restraint stress predicted longer FR2 escape latencies (Table 4.7).

Table 4.7 Standardized beta coefficients for physiologic variables predicting shuttle box escape responses

Physiologic Parameter	FR1	FR2
Cortisone	-0.06	0.02
Heart rate average	-0.27	-0.38
Heart rate STDEV	-0.06	-0.35
Heart rate bin 1	-0.22	-0.33
Heart rate bin 2	-0.23	-0.34
Heart rate bin 3	-0.21	-0.32
Heart rate bin 4	-0.38	**-0.48***

* $p < 0.05$

4.3.7 Stepwise discriminant analysis of physiologic measures

A stepwise discriminant analysis of heart rate average, heart rate standard deviation and corticosterone determined that no variable or combination of variables discriminated helpless from non-helpless subjects.

4.3.8 Bivariate Pearson correlations between physiologic parameters and open-field behavioral measures

The only variables that predicted helplessness vulnerability were novelty-specific activity and heart rate during the last 15 seconds of restraint stress. A covariance analysis showed that increased activity in the novel open field was significantly correlated with lower heart rate (Table 4.8). Specifically, novel open-field ambulation, short movement activity, rearing, and center time were all negatively correlated with heart rate. However, the same relationship was not evident with activity in the familiar open field. Only thigmotaxis in the familiar open field showed significant correlations with the third 15-sec heart rate bin. Corticosterone and heart rate variability did not show significant correlations with open-field behavior. Sucrose consumption measures did not show significant Pearson correlations with the physiologic parameters (r's ranged from -0.10 to 0.12). Likewise, latency to step down (r's ranged from -0.14 to 0.2) did not show significant Pearson correlations with physiologic parameters.

Table 4.8 Pearson correlations between behavioral and physiologic parameters.

				Heart Rate 15-Second Bins			
Behavioral Parameter	Cort	HR avg	HR stdev	HR1	HR2	HR3	HR4
Novel Open Field							
Ambulation Time (sec)	.22	**-.54***	.01	**-.50***	**-.47***	**-.51***	**-.53***
Short Movement Time (sec)	.04	**-.50***	-.09	-.41	-.39	**-.55***	**-.57***
Rearing Time (sec)	.02	**-.46***	-.15	**-.44***	-.40	**-.54***	-.42
Center Time (sec)	.00	**-.55***	.02	**-.45***	**-.44***	**-.59***	**-.65***
Rest Time (sec)	-.14	**.58***	.04	**.53***	**.50***	**.59***	**.63***
Familiar Open Field							
Ambulation Time (sec)	-.01	-.21	.00	-.20	-.16	-.22	-.22
Short Movement Time (sec)	-.02	-.36	.00	-.28	-.25	-.43	-.41
Rearing Time (sec)	.04	-.08	-.12	-.09	-.07	-.11	-.04
Center Time (sec)	.01	-.39	.00	-.31	-.32	**-.45***	-.40
Rest Time (sec)	-.08	.36	.00	.30	.27	.40	.39

***Significant correlation, p < 0.05; Cort, corticosterone; HR, heart rate; avg, average; stdev, standard deviation; HR1-4,15-second bins composing the one-minute sampling window.**

4.3.9 Principal component factor analysis

Novelty-specific activity and heart rate during the last 15 seconds of restraint stress were the only predictors of FR escape latencies. This raises the question of whether a combination of these two variables is a better predictor of escape latencies than either measure alone. First, a principal component factor analysis was performed to combine all novelty-specific indices into one score. A factor score was generated corresponding to one principal component of all novelty-specific ratios which could explain 78% of the variance in novelty-specific activity.

Multiple linear regressions analysis revealed that a higher novelty factor score and lower heart rate during the last 15 seconds of restraint stress significantly predicted greater FR1 and FR2 escape latencies ($p < 0.05$) and collectively explained 35% of the variance in FR1 and 41% of the variance in FR2 escape latencies. Therefore, a combination of these variables increased accountability for the variance in the FR2 escape response.

Furthermore, a discriminant analysis showed that the combination of a novelty factor score and heart rate during the last 15 seconds of restraint stress improved the predictability of helplessness susceptibility than using either measure alone ($\chi^2(2) = 9.0$, $p < .05$). Specifically, a combination of both variables correctly classified 86% of subjects into helpless and non-helpless categories versus a predictability of 76% with the novelty factor score ($\chi^2(1) = 5.9$, $p = .02$) and 67% with the last heart rate bin ($\chi^2(1) = 3.0$, $p = .08$).

4.4 DISCUSSION

4.4.1 The cluster analysis based on FR1 or FR2 escape responses is an unbiased classification tool

The learned helplessness paradigm (exposure to inescapable shock followed by escapable shock) may be a transient stressor, but it has profound effects on the behavior of subjects that show individual differences in susceptibility to acquiring helplessness. A novel finding of the present study was the categorization of subjects based on their susceptibility to helplessness. Previous studies have classified subjects as helpless when escape latencies are greater or equal than 20 s (Kram *et al.*, 2000; Petty *et al.*, 1993; Wieland *et al.*, 1986). However, these studies utilized five FR1 trials (to shape a behavioral response), followed by twenty-five FR2 trials. The FR2 trials are used as the contingency to determine helplessness, because normally, inescapably-shocked rats do not show escape deficits with the FR1 schedule (Hunziker & Dos Santos, 2007; Maier *et al.*, 1973). Previously in chapter 2, this same procedure was used - five FR1 followed by twenty-five FR2 trials - and we found that based on the FR1 contingency, 35% of our Holtzman male population was helpless and based on the FR2 response, 77% of the Holtzman males were helpless (Padilla *et al.*, 2009). In addition, even naïve Holtzman rats showed the same type of escape deficits with the FR2 schedule as inescapably shocked rats, a finding that has not been reported previously with other strains used in learned helplessness studies (Hunziker & Dos Santos, 2007; Maier *et al.*, 1973). Based on these results, we decided to modify the number of trials to fifteen FR1 and fifteen FR2 trials to obtain equal number of responses in each schedule. This improved the analysis and comparison of the subject's escape response in both schedules and reduced the amount of shock and animal distress. With the previous paradigm (5 FR1/ 25 FR2), a subject's cluster assignment could change depending on the type of escape response. However, with the new paradigm incorporating equal

numbers of FR1 and FR2 trials, our cluster analysis, which provides a mathematically unbiased method of sorting the subjects into distinct groups, separated the Holtzman population into 50/50 percent helpless/non-helpless, regardless of the FR schedule applied. A helplessness yield of 50 percent among Holtzman rats is similar to that obtained by other researchers with this strain (Wieland *et al.*, 1986; Kram *et al.*, 2000) and increases the validity of using 15 trials of each FR type.

4.4.2 Novelty-specific activity predicts susceptibility to helplessness

Prior to shock exposure, the detailed measurement of behavior can facilitate the characterization of these subjects before the helpless phenotype presents itself. Which individuals are more or less susceptible to the changes this transient stressor might cause? What "personality" traits, like novelty-evoked activity, reward dependence and harm avoidance might better predict this phenotype? In order to address these questions, we characterized open-field behavior, sucrose drinking and passive harm avoidance to evaluate predictors of helplessness.

Evaluating novel and familiar open field parameters can allow the differentiation of the following behaviors: novelty-specific activity, general hyperactivity, and faster habituation (Colorado *et al.*, 2006; Padilla *et al.*, 2009; Shumake *et al.*, 2005; Spivey *et al.*, 2008). For example, congenitally helpless rats showed hyperactivity during the first five minutes of an open field test (Vollmayr *et al.*, 2004). Furthermore, after re-exposing a subject to the same open-field chamber twenty-four hours later, Shumake et al. (2005) found that the behavioral activation among these rats was specific to a novel and not a familiar environment. Previous researchers have reported increased novelty reactivity based on one open-field session (Gilad & Shiller, 1989; Monzon & De Barioglio, 1999; Stead *et al.*, 2006; Vollmayr *et al.*, 2004), however, as depicted in Figure 4.2, one open field session is unable to distinguish between a subject that is truly hyperactive, excited by novelty or exhibits fast habituation learning. Schulz et al. (2010) reported that the

selectively-bred helpless strain showed a larger decrease in activity levels over trials indicative of enhanced habituation learning and not novelty-induced activity (Schulz *et al.*, 2010). The authors discussed that increased habituation may reflect decreased hedonic value of novelty or reduced escape attempts from the open field as another indicator of helpless behavior. However, their data are consistent with a habituation effect when simply contrasting helpless-susceptible vs. helpless-resistant lines, but when the selected lines are contrasted against wild-type rats, it is evident that resistant rats showed a pattern of hyperactivity (elevated activity relative to wild type in both novel and familiar open fields) and that susceptible rats showed a pattern of novelty reactivity (elevated activity relative to wild type in the novel but not familiar open fields). Novelty-evoked activity among helpless-susceptible animals in comparison to the non-helpless group is consistent with our results as shown in Figure 4.1.

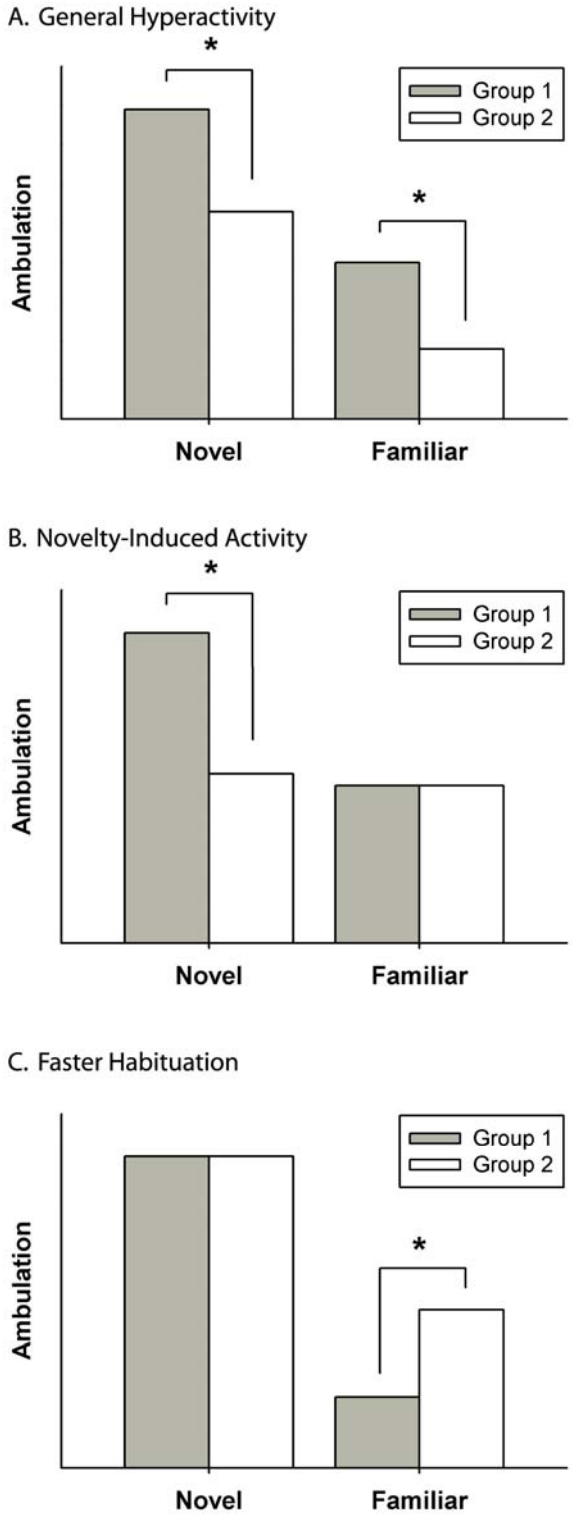

Figure 4.2 Schematic diagrams indicating the differences between general hyperactivity, novelty-induced activity and faster habituation as measured by the novel and familiar open field.

Furthermore, the only behavioral parameter that predicted susceptibility to helplessness was novelty-specific open field activity before exposure to the stress of learned helplessness training, signifying that animals predisposed to helplessness are likely to show greater novelty-induced exploratory activity. Cloninger proposed a system of personality description defined in terms of three dimensions: 1) novelty seeking, 2) reward dependence and 3) harm avoidance (Cloninger, 1987). Two studies using the tridimensional personality questionnaire or TPQ (based on Cloninger's system) reported that high novelty seeking, low reward dependence, and high harm avoidance were associated with PTSD symptomatology among Vietnam combat veterans (Richman & Frueh, 1997; Wang *et al.*, 1997), with higher novelty seeking being the strongest single predictor of PTSD severity (Richman & Frueh, 1997). However, Gil (2005) found the opposite among her sample of Israeli college students exposed to a terrorist attack: lower novelty-seeking scores before the attack predicted development of PTSD after the attack. There are a number of differences between the subjects of these studies which could account for the opposite findings: repeated combat stress over months to years versus an isolated traumatic event, an all male versus a mixed gender group, or a much younger and more educated group in the case of the Israeli study. Perhaps the most important difference, though, was the potential contribution of novelty seeking to trauma exposure in the case of the Vietnam veterans. That is, persons high in novelty seeking may seek out more dangerous situations and thus expose themselves to more psychological trauma, which then manifests as an increased incidence of PTSD. This could not have been a factor in the Israeli study as the traumatic event was a random explosion occurring near the subjects' university.

However, this self-selection cannot explain the relationship between high novelty exploration and helplessness susceptibility in the Holtzman rat population, since all of the subjects were exposed to the same stressor. In addition, Sprague Dawley rats

selectively bred for greater susceptibility to learned helplessness have a similar behavioral profile, characterized by increased exploration of novel environments (Shumake *et al.*, 2005). This supports the notion that novelty-induced activity is associated with a basic, physiological vulnerability to stress that is independent of the tendency of novelty-seeking individuals to expose themselves to higher levels of stress. As discussed previously (Shumake *et al.*, 2005), this vulnerability may involve reduced glucocorticoid signaling, which biases animals toward greater activity in the novel open field (Kabbaj *et al.*, 2000) and greater rates of learned helplessness (Papolos *et al.*, 1993). Indeed, high novelty seeking and low cortisol levels are reported covariates in veterans with PTSD (Wang *et al.*, 1997). A correlation between corticosterone and novelty-specific activity was not observed in our sample. It is possible that two to five minutes of restraint stress may not be enough to elicit a corticosterone response; however, congenitally helpless rats have shown reduced levels of corticosterone at baseline (Edwards *et al.*, 1999). Nonetheless, the behavioral characteristics shown by congenitally helpless and Holtzman rats suggest that the learned helplessness paradigm can be a useful tool for studying behavioral correlates and predictors of PTSD development.

The congenitally helpless rat also showed decreased sucrose consumption and increased behavioral inhibition or harm avoidant behavior compared to normal Sprague Dawley controls (Shumake *et al.*, 2005). Differences in these behavioral measures were not observed between helpless and non-helpless Holtzman rats. Even though helpless subjects spent more time drinking water compared to non-helpless, covarying for total water intake did not affect the results. Certain personality traits that predict helpless behavior may not emerge, unless they have been selected through many generations. Furthermore, the lack of difference on passive avoidance between groups may rule out altered pain perception and general learning disability as explanations of helplessness.

Novelty seeking was the single highest predictor of PTSD severity among combat veterans (Richman & Frueh, 1997), therefore this trait may be more strongly tied to the genetic predisposition to helplessness compared to other temperament characteristics. Notably, it was novelty-specific and not general activity that was predictive of helplessness. These results confirm previous observations with other Holtzman male cohorts that showed susceptibility to helplessness (Padilla *et al.*, 2009). However, our previous study also reported that increased general activity was predictive of shorter escape latencies among Holtzman females (Padilla *et al.*, 2009), but controlling for baseline differences in open field ambulation eliminated the sex difference in the escape response. This study only used male Holtzman rats; therefore results are consistent with our previous finding. Furthermore, both cohorts were measured during adolescence (postnatal day 40), in which rats are more exploratory and active (Shumake *et al.*, 2005; Spear, 2000). This may represent an ideal time to test novelty-induced activity and ambulatory behaviors in the open field.

Novelty seeking is also affiliated with certain comorbidities and sub-types of affective disorders (Kashdan & Hofmann, 2008; Mulder *et al.*, 1994). Specifically, high novelty seeking is associated with multiple substance abuse, including alcohol and nicotine (Batra *et al.*, 2008; Gabel *et al.*, 1999; Galen *et al.*, 1997; Wills *et al.*, 1995), and increased suicidality among patients with borderline personality disorder (McGirr *et al.*, 2007). In addition, high levels of novelty seeking, impulsivity, and sensation seeking were predictive of poor outcome and greater drop-out rates during treatment among patients with substance abuse problems (Staiger *et al.*, 2007). In a separate study, patients with low levels of novelty seeking, harm avoidance and reward dependence showed greater symptom improvement after a six week course of anti-depressant treatment (Joyce *et al.*, 1994). Compared to clinical and demographic variables, temperament was the only predictor which accounted for 50% of the variance in

treatment outcome among those severely depressed patients (Joyce *et al.*, 1994). Responsiveness to tricyclic anti-depressants also differed among depressed women; high harm avoidance predicted desipramine response while high reward dependence predicted clomipramine response (Joyce *et al.*, 1994). Based on the previous results, the proper assessment of personality traits can help guide decisions regarding disease management and improve treatment outcomes.

4.4.3 Heart rate as a physiologic predictor of helplessness among Holtzman rats

Variations in heart rate and blood pressure reflect activity and responsivity of the autonomic nervous system and provide a measure of the functional state of central neurobiological regulatory mechanisms, and individual differences in the physiological responses to stress (Jemerin & Boyce, 1990; Marchei *et al.*, 2009; Sheps & Sheffield, 2001). For example, diminished heart rate variability has been observed among depressed subjects when compared to non-depressed patients and reduced heart rate variability has been linked to a worse prognosis in patients with coronary artery disease (Sheps & Sheffield, 2001). Negative mood states have also been linked to autonomic nervous system disturbances and cardiac events (Schwarz *et al.*, 2003).

Heart rate measurements did not show mean differences between helpless and non-helpless Holtzman rats. However, decreased heart rate during the last fifteen seconds of restraint stress predicted increased susceptibility to helplessness and a combination of this heart rate measure with a novelty factor score significantly explained over 35 percent of the variance in escape latencies. Furthermore, a lower heart rate correlated with increased activity in the novel open field. This could represent a diminished sympathetic tone or elevated vagal tone present in animals that display greater novelty-induced behavioral activation. For example, novelty seeking was associated with lower heart rate among men and women (Puttonen *et al.*, 2008). Disruptions in the HPA and autonomic nervous system which regulate heart rate and

response to stress have been linked to depression and anxiety in humans and animals (Baker *et al.*, 1999; Jemerin & Boyce, 1990; King & Edwards, 1999; Marchei *et al.*, 2009; Pariante, 2009; Sheps & Sheffield, 2001; Shumake & Gonzalez-Lima, 2003). Whether the effects on heart rate represent individual differences in response to a stressor or a disruption in these regulatory mechanisms is presently unknown. However, novelty-specific activity and increased susceptibility to helplessness seem to be common factors in the physiologic difference observed and the relationship between behavioral and physiological traits provides an additional way of assessing predictors of helplessness susceptibility among the Holtzman strain.

Our finding that novelty-induced exploratory activity predicts helplessness is consistent with literature linking combinations of personality traits with a predisposition to post-traumatic stress disorder in humans (Richman & Frueh, 1997; Wang *et al.*, 1997), and also with previous work with the congenitally helpless rat (Shumake *et al.*, 2005). The present finding indicates that increased novelty-induced activity observed in congenitally helpless rats is not an artifact of selective breeding for helplessness susceptibility, but is already present in an unselected population of rats. This is the first study to show that the novel and familiar open field test can serve as an innocuous screening tool in the selection of candidates predisposed to helplessness. For researchers interested in maximizing their yield of helpless animals, this 2 day, ten-minute screening could save time and minimize the total number of animals subjected to shock. For example, according to the discriminant analysis, 7 out of 9 subjects with novelty-specific average velocity ratios greater than 1.2 should become helpless. One should note, however, that this criterion has only been established for male rats from the Holtzman strain, and it may not generalize to females or rats from other strains. Physiologic traits such as heart rate also seem to have some predictive value in relation to novelty-induced activity and helplessness susceptibility. In conclusion, increased

novelty-induced open-field activity and low heart rate appear to be markers of helplessness susceptibility, and this finding has interesting practical and theoretical implications for those wishing to model the predisposition to PTSD.

Chapter 5 Networks underlying helplessness in Holtzman rats

Learned helplessness in animals is a model of comorbid disorders such as depression and post-traumatic stress disorder. Even though stress plays a role in these disorders, there is a need to investigate neurobiological predispositions because not all subjects develop learned helplessness following the same stress. The objective was to compare the brains of Holtzman rats that show vulnerability or resistance to become helpless after the same behavioral treatment to identify the underlying brain differences. Brains were analyzed for regional metabolism and functional connectivity using quantitative cytochrome oxidase histochemistry, and examined for inter-regional brain correlations. Helpless rats showed elevated metabolism in the lateral habenula as compared to non-helpless rats. Opposite functional relationships between the infralimbic cortex and other prefrontal and mesolimbic regions also distinguished helpless from non-helpless rats. Finally, reduced functional connectivity among prefrontal cortex regions was pervasive in the helpless rats. It was concluded that the brain differences underlying the susceptibility to helpless behavior involve alterations in three major networks: 1) hypermetabolic lateral habenula, and 2) altered nigrostriatal and mesolimbic systems, and 3) reduced functional connectivity among prefrontal-limbic circuits. Changes within these brain networks and their interactions may provide new insights into potential targets to treat subjects with susceptibility to stress-related psychopathologies.

5.1 INTRODUCTION

Learned helplessness (LH) represents a failure to exhibit an escape response after exposure to inescapable stress (Overmier & Seligman, 1967). This paradigm serves as a useful tool to model stress-induced psychopathology, such as depression or post-traumatic stress disorder (PTSD) (Foa *et al.*, 1992; Petty *et al.*, 1996; Petty *et al.*, 1997; Seligman, 1975a). However individuals vary widely in how they respond to stress

emotionally, behaviorally and physiologically (Jemerin & Boyce, 1990; Marchei *et al.*, 2009; Pariante, 2009; Schmidt *et al.*, 2008). Hence there is a need to identify biological factors underlying vulnerability to helplessness that may be relevant to stress-induced psychopathology. Researchers have previously verified that the learned helpless protocol reliably discriminates between helpless and non-helpless rats (Drugan *et al.*, 1989; Petty *et al.*, 1994). For example, the Holtzman rat strain showed a bimodal distribution in susceptibility to helplessness, with about half of the rats becoming helpless while the other half remained non-helpless after the same training for acquisition of learned helplessness (Chapter 4). Therefore, the objective of the present study was to determine regional brain differences between helpless and non-helpless Holtzman rats, including metabolic brain mapping and analysis of functional connectivity differences.

Brains were analyzed for regional metabolism and functional connectivity using quantitative cytochrome oxidase histochemistry (Gonzalez-Lima & Cada, 1998) and examined for interregional brain correlations (Puga *et al.*, 2007). Metabolic activity was examined using cytochrome oxidase histochemistry because cytochrome oxidase is the terminal respiratory enzyme in the mitochondrial electron transport chain that is correlated to ATP synthesis and serves as an endogenous metabolic marker for neuronal functional activity (Wong-Riley, 1989). Cytochrome oxidase is unique in that it marks cumulative, long-term neuronal activity, thus making it ideal for evaluating regional brain differences between rats vulnerable or resistant to develop learned helplessness. This approach has also been used successfully to map the brain of congenitally helpless rats, a selectively bred line of Sprague-Dawley rats that displays spontaneous helpless behavior (Shumake & Gonzalez-Lima, 2003).

5.2 MATERIALS AND METHODS

5.2.1 Subjects

Subjects were 21 male Holtzman rats obtained from Harlan (Madison, WI) at postnatal day 30 (PD 30). Animals were housed 2–3 per cage and maintained on a 12 h/12 h light/dark photoperiod in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Food and water were available *ad libitum*. Subjects were handled and weighed for 5 minutes every day for 9 days prior to starting behavioral experiments. All experiments occurred during the light phase between 0700 h and 1900 h. Experiments were done in accordance with NIH guidelines for the use of experimental animals and were approved by the University of Texas Institutional Animal Care and Use Committee.

5.2.2 Behavioral experiments

Animals were the same subjects used in the behavioral experiments described in Chapter 4 (Table 5.1).

5.2.3 Brain tissue processing

On postnatal day 46, following decapitation, brains were removed and frozen rapidly in isopentane. Using a cryostat (Reichert-Jung) at -20°C, brains were sectioned at 40 µm and kept frozen at -40°C until they were processed using quantitative cytochrome oxidase histochemistry (Gonzalez-Lima & Cada, 1998), as described previously in Chapter 2.

Table 5.1 Experimental Design

Experiment	Postnatal day (PD)
Handling	31-39
Novel open field	40
Familiar open field	41
Sucrose test I (baseline)	41
Sucrose test II	42
Passive avoidance I (step-downs)	42
Passive avoidance II (no shock)	43
Learned helplessness training	43
Learned helplessness testing	44
Heart rate measurement	46
Trunk blood collection (corticosterone assay)	46
Decapitation/brain tissue collection	**46**

5.2.4 Regions analyzed

Regions of interest included: the prefrontal cortex (measured from superficial and deep cortical layers and including separate measures of prelimbic, dorsal, lateral, anterior insular, orbital ,infralimbic and anterior cingulate cortices), the medial and lateral nuclei of the habenula, the basal ganglia (caudate-putamen measured from anterior and posterior levels, nucleus accumbens shell and core, ventral pallidum, globus pallidus, substantia nigra pars compacta and reticulata), the extended amygdala (anterior, basolateral, central, medial nuclei and bed nucleus of stria terminalis), septal regions (lateral nuclei and medial septum/diagonal band), paraventricular hypothalamic nucleus, hippocampal regions measured from anterior and posterior levels (CA1, CA2, CA3, subiculum, and dentate gyrus), the interpeduncular nucleus, ventral tegmental area, dorsal raphe nucleus, median raphe nucleus, periaqueductal gray and the retrosplenial cortex. Regions of interest were selected based on previous research from our lab (Shumake *et al.*, 2000; Shumake *et al.*, 2004) and depression/PTSD neuroimaging studies (Freo *et al.*, 2008; Mayberg *et al.*, 1999; Mayberg *et al.*, 2000; Hull, 2002; Rauch *et al.*, 2006).

Sensory and motor regions that were not expected to show changes were also sampled: sensory regions related to olfaction (lateral nucleus of the olfactory tract), audition (dorsal inferior colliculus), vision (lateral geniculate nucleus), and somatosensation (lateral and medial nuclei of the ventral posterior thalamus); brainstem motor regions (pedunculopontine tegmental nucleus, pontine reticular nucleus, reticulotegmental nucleus); cortical regions (primary somatosensory cortex, entorhinal, perirhinal, and prepyriform cortex), and one white matter region (mammillothalamic tract).

5.2.5 Statistical Analyses

Two-step clustering analysis was applied to identify how subjects' escape latency in the shuttle box test differentiated them into distinct groups or clusters (Chourbaji *et al.*, 2005). This analysis calculated the dissimilarity or the distance between individuals based on their escape response, and segregated into two clusters animals that were helpless and non-helpless.

The cytochrome oxidase data sets were analyzed as follows. Regions were divided into subgroups. Repeated measures ANOVA were performed on each subgroup, followed by simple-effects tests as necessary. One-way ANOVA's were also performed on individual regions. A step-wise discriminant analysis was also performed, as used by McIntosh and Gonzalez-Lima (1994), to identify regions which might differentiate the experimental groups based not on mean differences, but on interregional covariance differences.

Functional connectivity was assessed using pair-wise correlations. Pearson product-moment correlations between all measured regions were calculated for each group (within-group analysis). A jackknife procedure was performed in which each individual subject was dropped from a group, and then correlations were calculated again without that subject's data. This procedure was iterated until each subject had been sequentially dropped and the analysis repeated. To minimize Type I error, only the correlations that remained significantly different from zero at $p < 0.05$ through all iterations were considered statistically reliable for further analysis. These correlations were then tested for significant differences between groups (between-group analysis). The Fisher Z transformation was used to convert each correlation to a Z score to test differences in regional correlations between groups (Bruchey & Gonzalez-Lima, 2006; Jones & Gonzalez-Lima, 2001; Puga *et al.*, 2007). The term functional connection or functional coupling was used to refer to a significant correlation between two brain

regions. Based on data from the jackknife correlations, a color correlogram was developed using Matlab software version 7.0 (Nair *et al.*, 2001).

5.3 RESULTS

A cluster analysis based on FR1 and FR2 escape responses divided the subjects into two distinct clusters with high and low escape latencies. These were termed the helpless and non-helpless clusters (Chapter 4).

A multivariate analysis of variance showed no significant difference on overall brain metabolic activity between helpless and non-helpless groups ($p = 0.14$). The significant regional effects are summarized in Table 5.2.

Table 5.2 Regional mean cytochrome oxidase activity (μmol/min/g) between helpless and non-helpless Holtzman rats

Region	Bregma (mm)	Helpless Mean ± S.E.	Non-helpless Mean ± S.E.
Habenula			
Medial habenula	-3.8	191.3 ± 4.2	180.4 ± 4.7
Lateral habenula medial	**-3.8**	**216.1 ± 4.5 ***	**196.0 ± 6.0**
Lateral habenula lateral	-3.8	222.0 ± 5.9	208.6 ± 8.2
Basal Ganglia			
Substantia nigra compacta	**-5.3**	**145.8 ± 3.9 ****	**160.7 ± 1.9**
Substantia nigra reticulata	-5.3	162.6 ± 4.0	173.1 ± 5.7
Anterior ventral pallidum	**0.7**	**214.4 ± 7.5 ***	**191.7 ± 6.5**
Posterior ventral pallidum	-0.3	171.0 ± 16.2	145.2 ± 5.4
Extended Amygdala			
Anterior amygdala	-1.3	217.9 ± 18.8	185.6 ± 20.0
Basolateral amygdala	**-2.3**	**220.3 ± 5.6 ***	**205.0 ± 3.6**
Central amygdala	**-2.3**	**216.3 ± 1.8 ***	**203.1 ± 4.8**
Medial amygdala	-2.3	200.8 ± 6.4	187.5 ± 5.1
Bed nucleus stria terminalis	-0.3	185.9 ± 8.9	165.9 ± 9.1
Dorsolateral-Cingulate PFC			
Dorsal frontal sup	3.7	192.3 ± 4.4	183.6 ± 4.7
Dorsal frontal deep	**3.7**	**205.6 ± 2.5 ***	**194.8 ± 3.7**
Lateral frontal sup	3.7	203.1 ± 3.6	190.5 ± 4.9
Lateral frontal deep	3.7	203.4 ± 4.1	192.6 ± 4.4
Anterior cingulate sup	**0.7**	**218.5 ± 6.9 ***	**202.1 ± 3.0**
Anterior cingulate deep	**0.7**	**219.1 ± 7.5 ***	**201.6 ± 3.1**
Limbic PFC			
Anterior insular sup	**3.7**	**191.9 ± 3.1 ***	**177.2 ± 6.0**
Anterior insular deep	3.7	194.3 ± 3.4	184.3 ± 4.7
Medial orbital sup	3.7	200.5 ± 3.6	189.9 ± 4.4
Medial orbital deep	**3.7**	**201.9 ± 2.3 ***	**188.9 ± 4.3**
Lateral orbital sup	3.7	203.4 ± 3.2	197.4 ± 5.6
Lateral orbital deep	3.7	196.2 ± 1.9	186.8 ± 4.9
Prelimbic sup	3.7	178.5 ± 3.4	171.8 ± 3.6
Prelimbic deep	3.7	183.9 ± 3.8	174.8 ± 4.7
Infralimbic sup	2.2	212.0 ± 5.0	206.6 ± 6.6
Infralimbic deep	2.2	217.8 ± 5.2	216.2 ± 6.1

PFC (prefrontal cortex), sup (superficial), * $p < 0.05$, ** $p < 0.01$

5.3.1 Habenula

There was a significant metabolic increase in the medial aspect of the lateral habenula among helpless animals compared to non-helpless (Table 5.2).

5.3.2 Basal Ganglia

Helpless subjects had significantly lower metabolic activity in the substantia nigra pars compacta compared to non-helpless. Helpless subjects had significantly higher metabolic activity in the anterior ventral pallidum compared to non-helpless (Table 5.2). Regional group differences were not identified in the nucleus accumbens shell and core, globus pallidus, or caudate-putamen ($p > 0.1$).

5.3.3 Extended amygdala

Repeated measures ANOVA of amygdalar regions (anterior, basolateral, central, medial and bed nucleus of stria terminalis) showed an overall significant group difference, $F(1,16) = 6.13$, $p = 0.03$. A one-way ANOVA of individual regions in the extended amygdala showed significant group differences with the basolateral and central nuclei of the amygdala (Table 5.2).

5.3.4 Prefrontal cortex

Helpless animals had increased metabolic activity in the anterior cingulate, the superficial layers of the anterior insular cortex and the deep layers of the dorsal frontal and medial orbital cortex (Table 5.2).

5.3.5 Other regions

A one-way ANOVA showed increased cytochrome oxidase activity in the somatosensory cortex of helpless rats ($p = 0.02$). This region was not expected to show a group difference; however, since helpless subjects had higher escape latencies and

consequently received more shock compared to non-helpless rats, increased sensory stimulation could be responsible for a hypermetabolic somatosensory cortex. Other regions including the raphe nuclei, periaqueductal gray, interpeduncular nucleus, septum and hippocampus did not show significant mean group differences ($p > 0.05$).

5.3.6 Stepwise Discriminant Analysis

A stepwise discriminant analysis was performed of all regions that showed significant group differences to determine which region, or combination of regions, best discriminates helpless from non-helpless subjects based on interregional covariance differences rather than mean differences. The final discriminant function included only two regions, the substantia nigra pars compacta and the deep layers of the anterior cingulate cortex, which classified 20 out of 21 subjects correctly ($\chi^2(2) = 15.72$, $p < .01$). This indicated that cytochrome oxidase activity in these two regions was the best predictor of helplessness susceptibility and that inclusion of additional variables did not improve predictability.

5.3.7 Subcortical interactions

The accumbens shell and ventral pallidum showed strong positive correlations with the septum among helpless subjects, and weak positive correlations or uncoupling between these regions was observed among non-helpless animals (Table 5.3). In general, strong functional couplings between regions that were observed among helpless subjects while the opposite relationship was observed among non-helpless animals, included: 1) the interpeduncular nucleus with the ventral tegmental area, dorsal raphe and anterior hippocampus and 2) the subiculum with the posterior hippocampus.

Strong interregional functional coupling among non-helpless subjects, while the opposite relationship was observed among the helpless group included: 1) the lateral

habenula with the anterior dentate gyrus and 2) the anterior caudate putamen with the dorsal raphe and periaqueductal gray.

In addition, intraregional subcortical connectivity was greater among non-helpless subjects. Specifically, the posterior hippocampus (CA1 and CA3), and the medial and ventral aspects of the bed nucleus of stria terminalis showed strong positive correlations with each other among non-helpless animals but were much weaker in the helpless group.

Table 5.3 Subcortical regional brain correlations: a comparison between helpless and non-helpless groups.

	ACB s		VP		CPU a		SUB		CA1 p		BST m		LHb l		IPN	
	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH
DG a	0.07	.43	.07	.44	.13	-.02	.17	.59	.05	.53	-.13	.07	**-.06***	**.80***	.59	.00
CA1 a	-.01	.08	-.03	.07	-.33	.08	-.13	.32	.01	.18	-.51	.05	.24	.82	**.85***	**-.04***
CA2 p	.10	-.01	.50	-.04	.01	.01	**.91***	**.21***	.89	.85	-.15	-.02	.04	.12	.04	.27
CA3 p	.08	-.05	.30	.03	.00	-.01	.79	.49	**.66***	**.95***	-.03	.02	.01	.08	.02	.06
LS	**.87***	**.03***	**.83***	**.04***	.23	-.04	.27	-.02	.13	.31	.12	.01	.03	.14	.24	.19
MSDB	.81	.46	**.91***	**.47***	-.03	-.38	.21	-.05	.25	.13	-.22	-.13	-.05	.05	-.03	-.12
BST v	.07	-.50	-.32	-.26	-.04	.37	-.26	.00	-.51	-.01	**.51***	**.97***	.00	.05	.01	.05
VTA	.29	.03	.05	.00	-.14	.00	-.04	-.10	.02	.00	-.36	-.40	.04	-.35	**.83***	**.00***
DR d	.18	.50	.10	.46	**.01***	**-.81***	.67	.00	.39	.02	-.19	-.47	.12	.08	**.53***	**-.47***
DR v	.18	.15	.07	-.06	**.00***	**-.89***	.55	-.04	.25	.01	-.21	-.47	.17	-.01	.30	-.45
PAG	.15	.38	.00	.24	**.01***	**-.89***	.40	.01	.13	-.01	-.18	-.40	.07	.01	.48	-.45

***Helpless vs. non-helpless, $p < 0.05$; a, anterior; p, posterior; d, dorsal; v, ventral; m, medial. DG, dentate gyrus. CA, hippocampus. LS, lateral septum. MSDB, medial septum/diagonal band. BST, bed nucleus of stria terminalis. VTA, ventral tegmental area. DR, dorsal raphe. PAG, periaqueductal gray. ACB s, accumbens shell. VP, ventral pallidum. CPU, caudate putamen. SUB, subiculum. LHb l, lateral habenula lateral aspect. IPN, interpeduncular nucleus.**

5.3.8 Cortical-subcortical interactions

The functional connectivity between cortical and subcortical regions also differentiated between helpless and non-helpless groups (Table 5.4). In general, among helpless subjects, the superficial layers of the prelimbic, dorsal frontal and medial orbital cortex showed strong positive correlations with the accumbens shell, ventral pallidum, medial septum/diagonal band, and lateral septum, which contrasted with the uncoupling or weak positive correlations between the same regions in the non-helpless group. Other strong functional connections in the helpless group included the interactions of the retrosplenial cortex with the dorsal raphe and periaqueductal gray.

The connection between the deep layers of the cingulate and the lateral habenula showed an opposite relationship between groups (a negative correlation among helpless and a positive correlation among non-helpless subjects). Non-helpless animals also showed strong negative coupling between the infralimbic cortex and the bed nucleus of stria terminalis as opposed to the non-coupling observed among helpless animals.

Table 5.4 Cortical-subcortical regional brain correlations: a comparison between helpless and non-helpless groups.

	PL s		DF s		MO s		IL s		IL d		Cg d		RS	
	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH
Acb s	**.85***	**.01***	.85	.42	.69	.44	.45	.42	.30	.29	.45	.26	.36	-.04
VP	**.87***	**.07***	**.95***	**.41***	.57	.42	.51	.17	.37	.06	.70	.13	.08	.00
MSDB	.73	.00	**.92***	**.19***	.56	.06	.46	.12	.36	.00	.54	.28	.25	.21
LS	**.83***	**.05***	.80	.08	**.77***	**-.06***	.67	-.01	.52	.07	.48	.24	.43	.28
BST m	-.18	-.08	-.03	-.64	.04	-.36	-.08	-.69	**.05***	**-.76**	.00	.00	.07	-.01
BST v	-.27	-.08	-.16	-.67	-.39	-.39	**-.03***	**-.79***	**.02***	**-.81**	-.08	.02	-.03	-.11
LHb l	-.02	.21	.08	.26	.07	.44	.18	-.06	.05	-.07	**-.38***	**.58***	.23	-.62
DR d	.26	.01	.14	.29	-.03	.21	.49	-.07	.54	-.01	.06	.16	**.82***	**-.26***
PAG	.22	-.12	.01	.02	.19	-.03	.39	-.10	.49	.03	.10	-.02	**.81***	**.01***

***Helpless vs. non-helpless, $p < 0.05$; s, superficial; d, deep; m, medial; v, ventral. ACB s, accumbens shell. VP, ventral pallidum. MSDB, medial septum/diagonal band. LS, lateral septum. BST, bed nucleus of stria terminalis. LHb l, lateral habenula lateral aspect. DR, dorsal raphe. PAG, periaqueductal gray. PL, prelimbic cortex. DF, dorsal frontal cortex. MO, medial orbital cortex. IL, infralimbic cortex. Cg, anterior cingulate cortex. RS, retrosplenial cortex.**

5.3.9 Prefrontal cortical interactions

Helpless subjects have more functional decoupling as evidenced by weaker negative and positive correlations between prefrontal cortical regions versus the non-helpless subjects which uniformly showed strong positive correlations (Table 5.5). The correlations between the superficial layers of the prefrontal cortex were illustrated using a correlogram (Figure 5.1). The findings from the correlogram are summarized as follows: Among helpless subjects, the anterior insular cortex showed correlations approximating zero or a functional decoupling to other prefrontal cortical areas as evidenced by the green squares. In addition, the infralimbic cortex showed opposite intercorrelations between groups. It was functionally decoupled from the lateral frontal and insular cortices in the helpless group, whereas, among non-helpless animals the infralimbic cortex was positively coupled to the lateral frontal and insular cortices and decoupled from the orbital and prelimbic cortices.

Table 5.5 Prefrontal cortical regional brain correlations: a comparison between helpless and non-helpless groups.

	DF s		DF d		LF s		LF d		AI s		AI d		LO s		LO d	
	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH	Help	NH
DF s	1.00	1.00														
DF d	**0.27***	**.88***	1.00	1.00												
LF s	.88	.96	.39	.85	1.00	1.00										
LF d	.66	.90	**.78***	**.98***	.75	.90	1.00	1.00								
AI s	**.12***	**.93***	**-.02***	**.77***	**.30***	**.90***	.42	.79	1.00	1.00						
AI d	**.33***	**.91***	.62	.88	**.16***	**.85***	.65	.92	.18	.82	1.00	1.00				
LO s	.54	.92	.59	.86	.43	.87	.75	.84	**.06***	**.87***	.60	.83	1.00	1.00		
LO d	-.44*	**.94***	**-.13***	**.84***	**-.37***	**.93***	**-.24***	**.87***	**-.29***	**.93***	**.09***	**.93***	**.19***	**.92***	1.00	1.00
MO s	.49	.88	.69	.90	.54	.84	.87	.87	.24	.78	.69	.78	.75	.94	**-.27***	**.84***
MO d	.00	.53	.06	.69	.15	.69	.35	.70	.86	.40	.26	.53	-.06	.60	**-.41***	**.60***
PL s	.82	.66	.64	.73	.70	.77	.85	.77	.33	.48	.74	.66	.70	.65	.20	.67
PL d	.68	.86	.76	.82	.67	.91	.92	.87	.33	.72	.76	.79	.66	.79	.29	.81
IL s	.46	.47	.27	.18	.20	.35	.42	.18	.05	.56	.65	.24	.61	.32	.12	.34
IL d	.31	.45	.19	.25	.02	.31	.15	.29	-.17	.54	.51	.37	.44	.37	-.02	.40

***Helpless vs. non-helpless, $p < 0.05$; s, superficial layer; d, deep layer. DF, dorsal frontal cortex. LF, lateral frontal cortex. AI, anterior insular cortex. LO, lateral orbital cortex. MO, medial orbital cortex. PL, prelimbic cortex. IL, infralimbic cortex.**

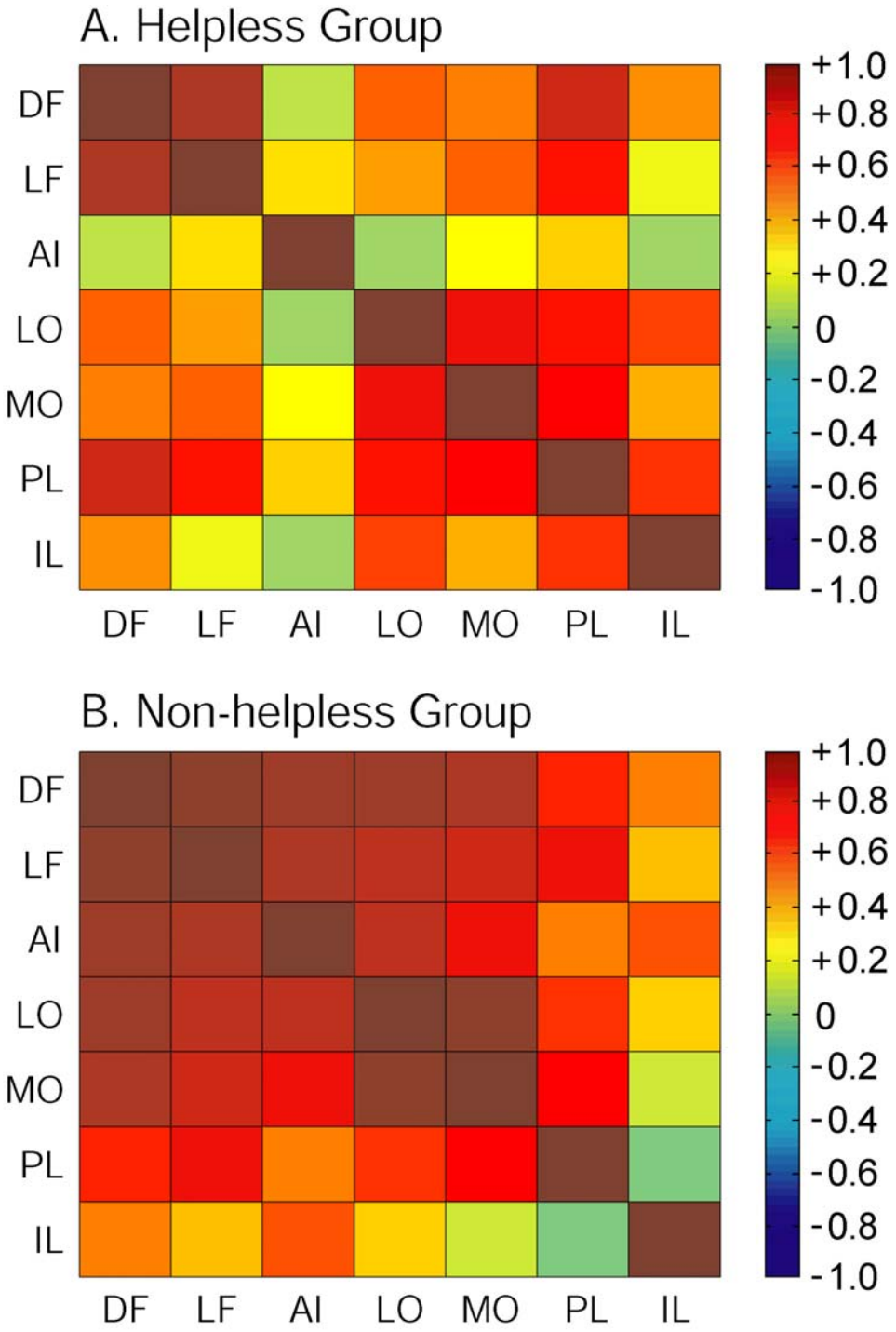

Figure 5.1 Correlogram of cortical interactions. The scale shows the relationship between positive and negative correlations. Positive correlations are represented as red and negative correlations are blue. Darker colors signify higher correlations. Only data from superficial cortical layers are depicted since we focused on cortical-cortical projections. DF (dorsal frontal), LF (lateral frontal), AI (anterior insular), LO (lateral orbital), MO (medial orbital), PL (prelimbic) and IL (infralimbic cortex).

5.4 DISCUSSION

The major findings of this study included: 1) elevated metabolism in the lateral habenula, 2) altered nigrostriatal and mesolimbic systems and 3) reduced functional connectivity among prefrontal–limbic cortical regions in helpless Holtzman rats.

5.4.1 Hypermetabolic habenula in helpless Holtzman rats

The lateral habenula has shown elevated metabolism in several animal models of depression: amphetamine withdrawal, chronic stress, α-methyl-para-tyrosine challenge (Caldecott-Hazard *et al.*, 1988) and the congenitally helpless rat (Shumake *et al.*, 2003). In addition, the habenula may play an important role in depression due to its implication in several processes affected in depression (see Chapter 1 for review). Indeed, habenula ablation completely blocked the development of learned helplessness in rats (Amat *et al.*, 2001). Furthermore, two human neuroimaging studies have shown increased habenula metabolism or blood flow correlated with depressive symptoms after acute tryptophan depletion (Morris *et al.*, 1999; Rosier *et al.*, 2009).

Notably, in our study increased habenular metabolism was restricted to the medial portion of the lateral habenula. The lateral habenula is composed of multiple subnuclei that are generally divided into medial (limbic) and lateral (motor) divisions (Andres *et al.*, 1999; Geisler & Trimble, 2008). The medial division receives afferents primarily from limbic regions that are directly or indirectly innervated by the cerebral cortex, the lateral hypothalamic and preoptic nuclei, basal forebrain structures including the ventral pallidum, substantia innominata, diagonal band nucleus, and the bed nucleus of the stria terminalis (Geisler & Trimble, 2008; Hikosaka *et al.*, 2008). Projections of the lateral habenula mainly target monoaminergic nuclei including the dopaminergic ventral tegmental area and substantia nigra pars compacta, and the serotonergic dorsal and

median raphe (Geisler & Trimble, 2008; Herkenham & Nauta, 1979). The net effect of cells from the lateral habenula is to inhibit dopaminergic neurons in the ventral tegmental area and substantia nigra (Christoph *et al.*, 1986; Shepard *et al.*, 2006). The habenula can also excite serotonergic neurons in the dorsal raphe with low frequency stimulation and inhibit these neurons with high frequency stimulation (Ferraro *et al.*, 1997).

Congenitally helpless rats showed increased metabolism in the habenula and decreased metabolism in the VTA and striatum (Shumake *et al.*, 2003) and helpless Holtzman rats also showed increased metabolism in the lateral habenula and decreased metabolism in the substantia nigra pars compacta. The habenula, through direct projections to these regions, could be inhibiting the dopaminergic pathways involved in reward and motor behaviors.

In addition, the lateral habenula is decoupled from the anterior dentate gyrus among helpless animals compared to non-helpless subjects. Reduced hippocampal volume and decreased neurogenesis within the dentate gyrus appear to play a role in major depression and response to antidepressants (Becker & Wojtowicz, 2007; DeCarolis & Eisch, 2010; Drew & Hen, 2007; Eisch *et al.*, 2008; Elder *et al.*, 2006; Gould & Tanapat, 1999; Kempermann & Kronenberg, 2003; Kempermann *et al.*, 2008). Less is known about the role of hippocampal function in depression (see Chapter 1 for review). Since stress-related psychopathologies are likely mediated by several regions in a neural network, identification of hippocampal functional interactions with regions that are also implicated in depression such as the lateral habenula (Morris *et al.*, 1999; Rosier *et al.*, 2009; Shumake *et al.*, 2003) may provide more information regarding the hippocampal mechanisms implicated in susceptibility to depression and response to antidepressants.

The interpeduncular nucleus (IPN) was positively coupled to the anterior hippocampus, ventral tegmental area and dorsal raphe among helpless animals, but it was uncoupled or showed the opposite relationship with these regions among the non-helpless group. The IPN projects to the hippocampus (Baisden *et al.*, 1979; Groenewegen & Steinbusch, 1984; Wirtshafter *et al.*, 1986), and raphe nuclei (Maciewicz *et al.*, 1981; Montone *et al.*, 1988). The IPN receives more acetylcholine input than any other region in the mammalian brain (Woolf & Butcher, 1985) and there is evidence that excessive cholinergic activity is implicated in the etiology of depression (Charles *et al.*, 1994; Dilsaver & Coffman, 1989; Janowsky *et al.*, 1983; Steingard *et al.*, 2000). Even though we cannot infer causality with the study of functional interactions, the positive functional coupling between the IPN and other subcortical regions in helpless rats could be associated with the cholinergic hyperactivity reported in depression studies. Additionally, a positive functional relationship between the IPN and the ventral tegmental area among helpless animals could be related to their parallel connections with regions that are involved in depression and helpless behavior such as the dorsal raphe and habenula (Contestabile & Flumerfelt, 1981; Herkenham & Nauta, 1979; Del Fava *et al.*, 2007; Groenewegen *et al.*, 1986; Hikosaka *et al.*, 2008; Lowry *et al.*, 2008; Maciewicz *et al.*, 1981; Montone *et al.*, 1988).

5.4.2 Nigrostriatal and mesolimbic pathways affected in learned helplessness

The substantia nigra pars compacta was the only region that showed hypometabolism among helpless rats compared to non-helpless subjects. In addition, activity in this region, and the deep layers of the anterior cingulate cortex, were a predictor of helplessness susceptibility. In humans, depression is the most common psychiatric comorbidity associated with Parkinson's disease, a neurodegenerative disorder characterized by dopamine cell loss in the substantia nigra pars compacta

(Marinus *et al.*, 2002; Okun & Watts, 2002). Similarities between psychomotor retardation in melancholic depression and Parkinson's disease include reduced motivation, slowness of thought, lack of spontaneity, inflexibility and impairment of attention (Lees, 1994; Rogers *et al.*, 1987). Psychomotor disturbance is associated with the melancholic subtype of depression which highly resembles the syndrome of the congenitally helpless rat (Shumake & Gonzalez-Lima, 2003). In the present study, reduced metabolic activity in the substantia nigra could potentially contribute to an escape deficit and increased susceptibility to helpless behavior in Holtzman rats.

In contrast to the substantia nigra, the ventral pallidum showed hypermetabolism among helpless animals. The ventral pallidum is a central convergence point for input from orbitofrontal, prefrontal and infralimbic cortex, amygdala, lateral hypothalamus, ventral tegmental area, parabrachial nucleus, subthalamic nucleus, and other structures related to reward (Carnes *et al.*, 1990; Chrobak & Napier, 1993; Fuller *et al.*, 1987; Klitenick *et al.*, 1992; Napier *et al.*, 1991; Reep & Winans, 1982; Saper & Loewy, 1980; Smith *et al.*, 2009b). Since orbitofrontal, prefrontal and amygdalar regions showed increased metabolism in helpless rats, it is possible that their projections could be associated to hypermetabolism in the ventral pallidum.

The ventral striatum (accumbens shell and ventral pallidum) showed strong positive coupling with septal regions (lateral and medial septum/diagonal band nuclei) among helpless subjects, and these functional connections were uncoupled or had weak positive correlations among non-helpless rats. Interestingly, reduced septal and accumbens metabolism has been reported among congenitally helpless rats (Shumake *et al.*, 2002; Shumake *et al.*, 2003) and data from learned helplessness experiments showed reduced c-fos activity in the lateral septum of rats that became helpless following inescapable shock (Steciuk *et al.*, 1999). A positive coupling between ventral

striatal and septal regions in helpless Holtzman rats may represent network dynamics that precede mean changes that occur in congenital helpless rats after many generations of selective breeding.

The dorsal striatum (caudate-putamen) showed functional uncoupling from the dorsal raphe and periaqueductal gray among helpless animals versus a strong negative coupling observed among non-helpless rats. Anatomical studies suggest bidirectional connectivity between the caudate putamen and dorsal raphe nucleus (Steinbusch *et al.*, 1981; Wictorin *et al.*, 1988). Furthermore, the dorsal raphe and periaqueductal gray are implicated in the expression of helpless behavior after inescapable stress (Grahn *et al.*, 1999; Maier *et al.*, 1993; Petty *et al.*, 1994) and regulation of defensive behavioral responses (Bandler *et al.*, 2000), respectively. Functional differences between nuclei that mediate reward and motor behaviors (ventral and dorsal striatum) with regions that are implicated in anxiety (septum) and stress-coping strategies (dorsal raphe and periaqueductal gray) may represent important neural mechanisms underlying the behavioral differences observed between helpless and non-helpless Holtzman rats.

Helpless Holtzman rats also showed increased amygdalar activity, especially in the central and basolateral amygdala. This is in contrast with the finding among congenitally helpless rats that showed decreased cytochrome oxidase activity in these regions. However, it is in agreement with human neuroimaging studies showing increased amygdala regional cerebral blood flow or glucose metabolism in depression (Drevets, 1999; Ho *et al.*, 1996; Nofzinger *et al.*, 1999). Furthermore, metabolic rate in the amygdala correlated with depression severity (Drevets, 1999) or negative affect (Abercrombie *et al.*, 1998) in depressed patients. However, it is also true that amygdala changes have not been observed in most studies and may only be present in patients with comorbid anxiety disorders (Davidson *et al.*, 2002). It has been hypothesized that at

baseline, the amygdala may be hypoactive, reflecting a generalized dopaminergic reward-processing deficit; but under conditions of stress, this area could become hyperactive, reflecting enhanced fear and anxiety (Shumake & Gonzalez-Lima, 2003). Since the behavioral characteristics of helpless rats are thought to overlap with symptoms of anxiety or post-traumatic stress disorder (Shumake *et al.*, 2005), the changes observed in the amygdala provide further support for use of the Holtzman strain in understanding the neural mechanisms underlying susceptibility to stress-related psychopathologies.

5.4.3 Helpless Holtzman rats showed reduced functional connectivity in the prefrontal cortex

Helpless subjects showed hyper-metabolism in prefrontal regions such as the dorsal frontal, insular, orbital and anterior cingulate cortex. However, the insular cortex showed decreased functional coupling with all other prefrontal regions and there were weaker positive correlations between dorsal frontal, lateral frontal and orbital cortices among helpless compared to non-helpless subjects. In the human neuroimaging literature on depression, abnormally decreased blood flow and metabolism in prefrontal areas are extensively replicated findings (Baxter, Jr. *et al.*, 1989; Bench *et al.*, 1992; Bench *et al.*, 1993; Mayberg, 2003; Videbech, 2000), although hyperfrontal activity has also been reported (Robinson *et al.*, 2008; Smith *et al.*, 2009a). Prefrontal hyper-metabolism may be an intraregional compensatory mechanism due to the lack of interregional communication. It has been hypothesized that frontal hyper-metabolism is an exaggerated or maladaptive compensatory process resulting in psychomotor agitation and rumination, attempting to override a persistent negative mood generated by abnormal chronic activity of limbic-subcortical structures (Mayberg, 2003). Nonetheless, reduced connectivity between prefrontal regions in the present study may

be indicative of a less interactive prefrontal cortex and may be more representative of the network mechanism underlying susceptibility to helplessness versus metabolic mean changes of individual regions.

Mayberg et al. (1997) proposed that limbic-cortical dysregulation is a model for depression. This model is characterized by dorsal cortical hypo-functioning with an overactive subgenual cingulate (also known as the infralimbic cortex in rodents). Interestingly, among non-helpless animals, all prefrontal regions showed positive correlations with each other except for the infralimbic cortex which showed positive correlations with the lateral and insular cortices, and functional decoupling from the orbital and prelimbic cortices. Among helpless subjects the relationship of the infralimbic cortex with these regions switched; the infralimbic cortex was positively correlated with the orbital and prelimbic cortices and was uncoupled from the lateral and insular regions. The subgenual/infralimbic cortex appears to have a role in modifying the activity of the autonomic nervous system (Bacon & Smith, 1993; Frysztak & Neafsey, 1991). Humans with lesions that include the subgenual cingulate show abnormal autonomic responses and blunted emotional reactions to emotionally provocative stimuli (Damasio *et al.*, 1990). Similarly, rats with infralimbic lesions show significantly reduced freezing and little or no ultrasonic vocalizations when presented with a conditioned stimulus predicting shock (Frysztak & Neafsey, 1991). Despite the lack of mean differences in the infralimbic cortex between helpless and non-helpless groups, the positive coupling of the infralimbic cortex with other ventral limbic areas (prelimbic and orbital cortices) and decoupling from lateral frontal structures among helpless animals could be a functional network alteration underlying the potential for increased emotionality and susceptibility to helplessness.

According to Mayberg et al., (1999), there is an inverse activity correlation between the dorsolateral prefrontal cortex and the subgenual cingulate in recovery from

chronic depression characterized by dorsolateral increases and subgenual cingulate decreases. One would expect such a negative activity correlation would exist among non-helpless rats. However, since the Holtzman strain has shown susceptibility to helplessness (Padilla *et al.*, 2009; Wieland *et al.*, 1986), non-helpless animals may not entirely represent a normal population. They may simply represent a more stress-resilient subgroup within a susceptible population and the positive functional coupling that exists between infralimbic and dorsolateral regions in non-helpless subjects could be mediated by connections that could potentially switch to a negative correlation with antidepressant treatment. Reduced connectivity between these regions among treatment naïve helpless animals might suggest that they lack the structural foundation to respond to antidepressants and they could represent a treatment resistant subgroup of the Holtzman population.

In addition, activity in the superficial layers of the prelimbic, dorsal frontal and medial orbital cortices showed strong positive correlations with the ventral striatum (nucleus accumbens shell and pallidum) and septum (medial and lateral nuclei) among helpless subjects. In contrast, non-helpless subjects showed a general decoupling or weak positive correlations between these cortical and striatal /septal regions. The main projection sites of the prelimbic cortex include: the nucleus accumbens, agranular insular cortex, basolateral nucleus of the amygdala, and the dorsal and median raphe nuclei of the brainstem, and these projections are consistent with a role of the prelimbic cortex in cognitive/limbic functions (Vertes, 2004). This region has shown both elevated and suppressed blood flow and metabolism in untreated depression (Baxter, Jr. *et al.*, 1989; Bench *et al.*, 1993; Drevets, 2000; Mayberg, 1994; Mayberg *et al.*, 1997), however, elevated activity predicted response to antidepressant treatment and suppressed activity predicted a poor response (Mayberg *et al.*, 1997).

Another region linked to depression and treatment response is the infralimbic cortex (Cosgrove & Rauch, 1995; Mayberg, 2003). In the current study, we found that infralimbic cortical activity showed a different relationship with the bed nucleus of stria terminalis between groups (uncoupled among helpless and negatively coupled among non-helpless). The main projection sites of the infralimbic cortex include: the bed nucleus of stria terminalis, lateral septum, preoptic nuclei, medial, central, and cortical nuclei of the amygdala, and hypothalamic nuclei (Vertes, 2004). The pattern of infralimbic projections is consistent with a role for the infralimbic cortex in the control of visceral/autonomic activity and stimulation of this region can result in changes in heart rate, blood pressure, and gastric motility (Hurley-Gius & Neafsey, 1986; Terreberry & Neafsey, 1987). In addition, activity in the infralimbic cortex or subgenual cingulate has correlated with response to antidepressants in humans (Mayberg *et al.*, 2000) and is a target for treatment resistant depression (Cosgrove & Rauch, 1995; Lozano *et al.*, 2008; Hamani *et al.*, 2010). The prelimbic and infralimbic cortices appear to have significant roles in antidepressant treatment response and identification of their functional interactions with subcortical regions that could also be affected in depression represent potential neural predictors of treatment response.

Activity in the anterior cingulate cortex was a predictor of helplessness susceptibility as evidenced by the discriminant analysis, and this region was hypermetabolic among helpless rats compared to non-helpless subjects. Furthermore, helpless and non-helpless subjects also showed the opposite relationship between the deep layers of the cingulate cortex and the lateral habenula (negative coupling among helpless and positive coupling among non-helpless).

In addition, metabolism in the retrosplenial cortex (also known as the posterior cingulate in humans) showed functional connectivity differences with the dorsal raphe

and periaqueductal gray. In humans, the posterior cingulate participates in tasks of emotional processing (Aleman, 2005; Malhi *et al.*, 2007). In rats, the retrosplenial cortex is involved in functions that include fear-based avoidance learning (Mello e Souza *et al.*, 1999; Souza *et al.*, 2002). The retrosplenial cortex receives projections from the dorsal raphe (Vogt *et al.*, 1979) and sends efferents to the periaqueductal gray (Van Groen & Wyss, 2003). These nuclei have roles in stress-induced helpless behavior (Grahn *et al.*, 1999; Maier *et al.*, 1993; Petty *et al.*, 1994), and the regulation of "fight-or-flight" and panic-like autonomic and behavioral responses (Bandler *et al.*, 2000), respectively. Based on these findings, functional differences in these cingulate-subcortical interactions could represent important mechanisms mediating fear-related behaviors and/or stress-induced helplessness.

In summary, the neural network mechanisms that underlie susceptibility to helpless behavior have been discussed in relation to other rat models of depression and human depression/PTSD literature. Several observations have been consistently implicated in these studies such as a hypermetabolic habenula, dysfunctional nigrostriatal and mesolimbic systems, and alterations in the prefrontal-limbic network. In addition, this study is unique because it examined networks underlying helpless behavior in Holtzman rats. Researchers can greatly benefit from the use of this strain without the need for selective breeding of the helpless trait over many generations. Characterizing the relationships between neurophysiological factors that have been extensively implicated in animal and human depression research may provide further insight into the functional changes that accompany susceptibility to stress-related psychopathologies.

Chapter 6 General Discussion

The main findings of this dissertation include: 1) the Holtzman rat represents a commercially-available model to study stress-related psychopathology; 2) individual differences in the response to fluoxetine permitted the characterization of networks underlying treatment response; 3) increased novelty-specific activity and reduced heart rate are predictors of vulnerability to helpless behavior; 4) elevated metabolism in the habenula, dysfunctional nigrostriatal and mesolimbic systems, and a less interactive prefrontal-limbic network are the neural mechanisms underlying helplessness vulnerability in the Holtzman rat.

Compared to diseases such as hypertension and diabetes, diagnosis and treatment of depression and anxiety do not commonly follow uniform algorithm guidelines based on the collection of objective neurophysiologic criteria. Generally, diagnoses of psychiatric disorders are determined by the presence of specific symptoms for a determined amount of time (American Psychiatric Association, 2000b). This scenario is further complicated by considerable symptom overlap between psychiatric disorders and heterogeneity of symptoms within disorders. For example, a patient with major depression can have weight loss or weight gain and hypersomnia or insomnia. However, mood disorders are not characterized by impairment in one system and generally encompass a dysfunction in distributed brain regions that can lead to various emotional, physiologic and somatic effects. Therefore, the presence of specific symptoms definitely play an important role in determining diagnosis and treatment of mood disorders, but there is a need to incorporate additional objective criteria that could potentially improve upon current practices. The long-term goal of this dissertation was to identify neurobiological mechanisms that underlie vulnerability to stress and response to antidepressant treatment. The overarching hypothesis is that animals with a genetic

predisposition to stress-induced helplessness will show behavioral and neurophysiologic patterns that are predictive of helpless behavior and treatment response. This line of research can potentially bring forth a new way of implementing systematic diagnostic and treatment plans.

6.1 HOLTZMAN RAT AS A COMMERCIALLY-AVAILABLE MODEL TO STUDY STRESS-RELATED PSYCHOPATHOLOGY

Holtzman rats showed worse escape latencies compared to two other rat strains, Sprague Dawley and Long-Evan rats. In addition, we found that Long-Evans rats, previously untested in the learned helplessness paradigm, were more similar to Holtzman rats in showing poor escape performance. However, of the three strains, Holtzman rats had the longest escape latencies and the highest percentage of failed trials. In general, however, strain differences in open-field behavior were minimal and could not account for the strain differences in escape latencies. This suggests that strain differences in escape latencies are due to an inherent susceptibility or resistance to helpless behavior unrelated to baseline locomotor behavior. While Sprague Dawley and Holtzman rats are distinct strains, it is likely that they share the majority of their genes because of their common ancestry. The Holtzman outbred rat, also known as Holtzman-Sprague Dawley, was originally developed from Sprague Dawley stock by the Holtzman Company in Madison, Wisconsin in 1947 and were later acquired by the Harlan Company in 1986. In this same year Weiland et al (1986), tested various inbred and outbred stocks of rats for susceptibility to learned helplessness, including Holtzman and Sprague Dawley outbred strains. Interestingly, I report similar results as he did 24 years ago: 20 percent of Sprague Dawley and over 50 percent of Holtzman rats acquired learned helplessness based on an FR2 response schedule.

Holtzman rats display different responses as compared to Sprague Dawley rats in other behavioral manipulations and drug treatments. Briefly, the Holtzman rat stock showed worse baseline performance on a delayed reinforcement task, had a blunted hypothermic response to a 5HT-1A receptor agonist (which is an effect observed in depressed patients that have a 5HT-1A receptor abnormality), and only the Holtzman strain improved their performance on the delayed reinforcement task after administration of a serotonergic antidepressant (Balcells-Olivero *et al.*, 1998). Another study reported that Holtzman differed from Wistar and Sprague Dawley albino strains in conditioned avoidance and that diazepam affected the avoidance behavior of Holtzman rats differently than Sprague Dawley rats—low levels of diazepam inhibited the avoidance response in Holtzman rats while enhancing it in Sprague Dawley rats (Kuribara *et al.*, 1976). Similarly, chemical lesions of the catecholaminergic system resulted in differing ethanol consumption rates—no change in Holtzman rats, but decreased consumption in Sprague Dawley rats (Melchior & Myers, 1976). Subtle genetic variations could be responsible for the divergent responses between Holtzman and Sprague Dawley albino strains that render them more or less susceptible to different behavioral phenotypes, particularly when confronted with stressful manipulations such as learned helplessness (Padilla *et al.*, 2009; Wieland *et al.*, 1986) or mother-infant separation (Spivey *et al.*, 2008). However, environmental factors cannot be ruled out as being responsible for the observed differences between these strains, especially in studies where they are obtained from different suppliers (Balcells-Olivero *et al.*, 1998). This is not true in my case, because I obtained both Holtzman and Sprague Dawley rats from the same supplier. Therefore, equal environmental conditions for the care of both strains are assumed. Knowledge of the genotypic and phenotypic differences between commonly used rodent strains can aid researchers at the time of choosing an animal model for

investigation of a specific phenomenon. The present work supports that the Holtzman strain is ideal for investigations related to helplessness vulnerability.

6.2 EXAMINING FST BEHAVIOR IN A STRESS-SUSCEPTIBLE MODEL

Previous studies have examined the effects of antidepressants using "normal animals" without *a priori* knowledge of susceptibility to helpless or depressive-like behavior (Cryan *et al.*, 2005a; Detke *et al.*, 1997; Porsolt *et al.*, 1977). Two studies reported that normal rats did not respond to antidepressant treatment in comparison to susceptible rats. Specifically, serotonergic antidepressants (fluoxetine and imipramine) did not improve the performance of Sprague Dawley rats in a delayed reinforcement task but they did improve responding among Holtzman rats (Balcells-Olivero *et al.*, 1998). The second study reported lack of improvement in FST-induced immobility after chronic 18 day antidepressant treatment among Sprague Dawley rats, however Flinders sensitive line rats (a depression animal model) showed an antidepressant response (Zangen *et al.*, 1997). Moreover, studies that showed reduced immobility in the FST with normal rat strains, generally used acute and/or supra-therapeutic doses of antidepressants (Bianchi *et al.*, 2002; Dow *et al.*, 2005; Jang *et al.*, 2009). Therefore, decreased immobility might be an anxiogenic response to increased monoamine brain concentrations that is unrelated to an antidepressant effect. In support of this statement SSRI's are known to produce anxiety in nondepressed humans and can be acutely anxiogenic among depressed patients (American Society of Health-System Pharmacists, 2001) and rodents (Liu *et al.*, 2010; Silva & Brandao, 2000).

Two studies did show reduced FST immobility with Sprague Dawley rats after 14 days of fluoxetine administered at lower doses (5mg/kg or less) (Cryan *et al.*, 2005a; Detke *et al.*, 1997). However, neither study assessed baseline and post-treatment locomotion to rule out excess monoamine-induced anxiogenesis or hyperactivity. To

address this concern one might subject the animal to an open-field test before and throughout drug treatment. If the animal fails to show habituation or has increased motor responses in the open field compared to baseline, this would suggest that the drug increased general locomotion independent of an antidepressant response. Furthermore, in the present work, vehicle and fluoxetine-treated Holtzman rats did not show behavioral differences in the post-treatment FST Day 1 session, suggesting that 1) decreased immobility in the Day 2 session is not mediated by anxiogenic responses to fluoxetine and 2) exposure to inescapable forced swim stress followed by a test session 24 hours later is an important determinant in the induction of helpless behavior and observation of a treatment effect. Based on these results, the behavioral improvement observed was due to an antidepressant effect which supported the use of the Holtzman strain to characterize the neural mechanisms underlying treatment response.

Moreover, vehicle-treated Holtzman rats had approximately a 10% increase in their immobility time after 2 weeks of antidepressant treatment compared to fluoxetine-treated subjects which showed a 20% improvement (i.e. less immobility time). Worsening of depressive-like behavior in vehicle-treated subjects is not unexpected in a stress-susceptible strain after two weeks of daily aversive intraperitoneal injections. Chronic injections with saline also increased FST immobility in the Flinders sensitive rat model of depression (Zangen *et al.*, 1997). Future studies could examine antidepressant effects using oral ingestion of fluoxetine containing appetitive food (e.g. vanilla wafers), which would not introduce additional stress to the animal and has increased translational relevance.

6.3 NOVEL AND TRANSLATIONAL APPROACHES TO EXAMINE THE EFFECTS OF ANTIDEPRESSANT TREATMENT

I analyzed the effects of fluoxetine treatment on forced swim behavior and brain function of Holtzman rats to further characterize this strain as a model of stress vulnerability. I provided evidence that the use of this strain can also model the existence of individual differences in treatment response rates, as occur in humans. Our novel approach for behavioral classification was able to differentiate between SSRI treatment responders and non-responders. This constitutes the first description of individual differences in treatment response to an antidepressant medication in an animal model that can further our understanding on the neuropsychopharmacology of depression. Hundreds of studies have utilized the FST to behaviorally evaluate antidepressant efficacy, yet none have distinguished responding from non-responding subjects. Part of the reason for this may be that other researchers have simply not looked for such individual differences. However, there are three unusual, if not unique, aspects to the methodology which may have contributed to this finding. First, the vast majority of FST studies have utilized acute (within a 24-hour period) administration at supra-therapeutic doses; only a few have utilized chronic administration at therapeutic doses (Cryan *et al.*, 2005a; Detke *et al.*, 1997). Second, to my knowledge, this is the first time treatment response in the FST has been operationalized in terms of test-retest improvement. Finally, I used a relatively uncommon rat strain which shows heightened stress vulnerability (Padilla *et al.*, 2009), which could also manifest as a resistance to antidepressant drugs.

Extensive genetic, behavioral and neurophysiologic evidence points to the fact that individual differences represent an important aspect of clinical psychopharmacology (Enns & Cox, 1997; Joyce *et al.*, 1994; Mayberg *et al.*, 2000; Nelson & Cloninger, 1997;

Pollock *et al.*, 2000; Rausch, 2005; Seminowicz *et al.*, 2004). However, this concept has been largely overlooked in animal research. In general, animal researchers focus on the detection of group mean differences in response to a specific treatment. Furthermore, the presence of individual differences in these studies is unfavorable because they can mask the detection of group effects. Individual variability is an aspect of pharmacology that should be readily acknowledged, especially since individual differences represent a critical component in the response to antidepressant treatment among humans.

6.4 A NETWORK OF FLUOXETINE TREATMENT RESPONSE

This work demonstrated distinct brain activity patterns between responders and non-responders to fluoxetine antidepressant-treatment. I believe this represents a step towards maximization of treatment efficacy based on neurobiological individual differences. After completion of the first study (Chapter 2), I discovered a bimodal distribution in escape responses that was skewed towards a higher percentage of helpless males. This could potentially account for the higher percentage of non-responders (61%) versus responders (39%). This percentage of non-responders is approximately twice the amount reported in depressed humans (Fava & Davidson, 1996). Genetic variations of neurotransmitter receptors, transporters and metabolic enzymes could represent possible structural mechanisms that underlie the differences in functional connectivity between treatment responders and non-responders (Lerer & Macciardi, 2002; Rosenzweig-Lipson *et al.*, 2007; Serretti *et al.*, 2002; Veenstra-VanderWeele *et al.*, 2000). For example, the short allele variant of the human serotonin transporter (SERT) gene has been associated with poorer responses to SSRI treatment among patients with depression (Durham *et al.*, 2004; Pollock *et al.*, 2000; Zanardi *et al.*, 2001), reduced transcriptional efficiency of the SERT gene promoter, decreased SERT expression and serotonin uptake (Lesch *et al.*, 1996), reduced functional coupling

between areas of the prefrontal cortex and subcortical regions (Pezawas *et al.*, 2005), and, alterations in frontal-limbic white matter microstructure (Pacheco *et al.*, 2009). Genotyping and metabolic brain mapping could be applied concurrently to determine structural and functional mechanisms underlying SSRI treatment response in this animal model. Nevertheless, I demonstrated that metabolic mapping techniques reveal network differences between responders and non-responders to antidepressant treatment.

Despite antidepressants being the most commonly prescribed drugs in clinical practice (Olfson & Marcus, 2009), not much is known regarding the network effects that underlie treatment response. Mayberg et al. (1997) proposed that limbic-cortical dysregulation underlies depression. This network model is characterized by dorsolateral cortical hypoactivity and hyperactive ventral medial limbic areas including the subgenual cingulate. Response to SSRI antidepressants in humans was characterized by a reversal of these effects as evidenced by increased metabolic activity in dorsolateral prefrontal cortex coupled with a reduction in the subgenual cingulate after six weeks of treatment (Goldapple *et al.*, 2004; Mayberg *et al.*, 2000). Furthermore, non-response to SSRI treatment was characterized by a failure to induce these adaptive changes (Mayberg *et al.*, 2000). To my knowledge, this is the first report of opponent functional coupling between ventral-medial and dorsal-lateral frontal regions related to successful antidepressant response in an animal model. I am excited by the convergence of this finding with the theory derived from human neuroimaging work.

Both prelimbic and infralimbic cortices appear to play major roles in predicting or determining response to antidepressant treatment and recovery from depression in humans (Dunn *et al.*, 2002; Goldapple *et al.*, 2004; Liotti *et al.*, 2000; Mayberg *et al.*, 1997; Mayberg *et al.*, 1999; Mayberg *et al.*, 2000). This work supports the importance of cortico-limbic interactions together with the dorsal raphe nucleus, habenula, and

interpeduncular nucleus in mediating the effects of an antidepressant response. These regions have been extensively implicated in depression research; however this is the first time a cortical-subcortical network differentiating responders and non-responders to fluoxetine treatment have been identified in an animal model.

Another interesting observation is the strong positive correlation between the dorsal raphe and interpeduncular nucleus among helpless-untreated subjects and fluoxetine non-responders. The fact that a strong positive influence from the interpeduncular nucleus to dorsal raphe was the only path in the structural equation model that specifically differentiated non-responders from responder and vehicle groups indicates that it may represent an important subcortical predictor of treatment resistance. Having obtained such a high yield of treatment non-responders, in addition to the identification of neural correlates of fluoxetine non-response, suggest that the Holtzman strain is a preferred tool to study the neural mechanisms underlying treatment resistance. Deep brain stimulation is a therapeutic intervention that has been applied successfully in treatment resistant depression among humans (Lozano *et al.*, 2008; Mayberg *et al.*, 2005), however the mechanisms underlying a response to this treatment are yet to be completely understood (Hamani *et al.*, 2010). Further investigation into these mechanisms can be performed using the Holtzman strain which showed increased predisposition towards antidepressant treatment resistance. This line of research could be of great clinical value since establishing a network that predicts treatment response can potentially lead to routine screening of candidates for specific types of treatment interventions.

6.5 PREFRONTAL CONNECTIVITY INDICATES STRESS VULNERABILITY AND RESPONSE TO ANTIDEPRESSANTS

Prefrontal hyperactivity coupled with reduced functional connectivity was observed among helpless Holtzman rats. Even though reports of decreased blood flow and metabolism in prefrontal areas are extensively replicated findings in the human literature (Baxter, Jr. *et al.*, 1989; Bench *et al.*, 1992; Bench *et al.*, 1993; Mayberg, 2003; Videbech, 2000), hyperfrontal activity has been reported as well (Robinson *et al.*, 2008; Smith *et al.*, 2009a). However, there is less investigation into the covariance relationships between brain regions as an indicator of functional connectivity. For example, in the present work, the infralimbic cortex seems to play an important role in treatment response, given that it is functionally decoupled from all other prefrontal areas among fluoxetine non-responders (Chapter 3). The insular cortex showed a similar pattern among helpless subjects, suggesting a role for this region in stress vulnerability (Chapter 5). In addition, the opposing relationship of the infralimbic cortex with the lateral-insular and prelimbic-orbital regions could also be related to stress vulnerability. It is unlikely that complex psychopathologies are mediated by regional changes in isolation of a neural network; therefore impaired communication between key structures may be more representative of the mechanisms underlying vulnerability to stress-induced helplessness versus observation of isolated regional changes in metabolic activity.

6.6 THE HABENULA AS AN IMPORTANT THERAPEUTIC TARGET

Helpless Holtzman rats showed hypermetabolism in the lateral habenula which has been a consistent finding with other animal models of depression (Caldecott-Hazard *et al.*, 1988) including the congenitally helpless rat (Shumake *et al.*, 2003). In addition, habenula activation appears necessary for the development of learned helplessness

(Amat *et al.*, 2001). Furthermore, two human neuroimaging studies have shown increased habenula metabolism or blood flow correlating with depressive symptoms (Morris *et al.*, 1999; Rosier *et al.*, 2009). In the present work, fluoxetine responders and non-responders showed an opposing relationship between the lateral habenula and the dorsal raphe (Chapter 3). Interestingly, the mRNA for an orphan G-protein-coupled receptor (GPCR-2037) was shown to be exclusively expressed throughout the medial and lateral habenula (Berthold *et al.*, 2003). It is termed an "orphan" because its ligand has not been identified. Future studies into the structure and function of this receptor could provide a way of selectively targeting the habenula with a systemically administered drug. Collectively, these findings support the role of the habenula as an important target for treatment of depression.

6.7 SUBCORTICAL CORRELATES OF HELPLESSNESS

PET and fMRI have given researchers the ability to map the living human brain, providing a wealth of data on all manner of psychiatric disorders, including depression. Since these studies almost always measure blood flow or metabolism, analogous neuroimaging techniques in animal models may provide an excellent bridge to the human disorders they are purported to simulate. Metabolic mapping techniques can provide a common brain measure for investigating apparent behavioral similarities. Furthermore, since current imaging technology cannot localize signals to specific subcortical nuclei in humans, metabolic brain mapping using animal models provides a means for speculating about functional activity in these undetectable regions of humans. If a model shares a pattern of cortical activity with a human disorder, one may speculate that the animal's subcortical pattern of activity would be shared as well. For example, in the present work, knowledge of the metabolic activity in the substantia nigra could be used to predict helplessness vulnerability among Holtzman rats.

6.8 PREDISPOSITION TO LEARNED HELPLESSNESS OR CONSEQUENCES OF INESCAPABLE STRESS

It is worth mentioning that the neural effects in the Holtzman rat were observed *after* the stress of learned helplessness training. Even though cytochrome oxidase is a long-term marker of metabolic capacity (Gonzalez-Lima & Cada, 1998; Wong-Riley *et al.*, 1998), there have been drug-induced changes in CO activity as early as six hours after treatment (Gonzalez-Pardo *et al.*, 2006). Therefore, I cannot infer causality from observed changes in metabolic activity and functional connectivity, since these effects could have been induced by the stressor itself. In future studies, stress-naïve Holtzman rats could be compared to Sprague Dawley rats, which showed resistance to helplessness (Padilla *et al.*, 2009; Wieland *et al.*, 1986). Comparisons between these strains that share a common ancestry may reveal additional information in a way that is comparable to observations with congenitally helpless and non-helpless rats. Furthermore, these comparisons can be performed without the burdensome task of selective breeding which can take years.

Stress-naïve animals provide a way of distinguishing between changes present at baseline versus those that were induced by the stressor. This might have practical clinical applications when considering an individual whose pathology has been triggered by a traumatic event versus a person with endogenous depression. There is extensive symptom overlap across various mood disorders such as major depression and PTSD. Even though these conditions are treated with similar pharmacotherapeutic agents, it is likely that distinct network mechanisms are involved in the pathology, and could play a significant role in treatment selection. For example, a proposed neurocircuitry model of PTSD is characterized by amygdala hyperresponsivity, reduced ventral medial prefrontal cortex function and elevated hippocampal activity (Rauch *et al.*, 2006). This particular

neural pattern may be more responsive to one type of treatment over another. Knowledge of the mechanisms that are unique to individual patients may help clinicians empirically determine adequate treatment plans and reduce the rate of non-response to psychotropic treatment.

6.9 NOVELTY-SPECIFIC ACTIVITY AND HEART RATE ARE BIOBEHAVIORAL PREDICTORS OF SUSCEPTIBILITY TO HELPLESSNESS AMONG HOLTZMAN RATS

Using two distinct cohorts (Chapters 2 and 4), we replicated the finding that increased novelty-specific activity prior to stress exposure constitutes a significant predictor of stress vulnerability. These results support previous studies showing that congenitally helpless rats showed increased novelty-induced activity compared to normal Sprague-Dawley controls (Shumake *et al.*, 2005). Novelty-evoked activity and vulnerability to helplessness have been linked to a dysfunctional HPA axis since high novelty responding rats and congenitally helpless rats have elevated PVH activity (Kabbaj & Akil, 2001; Shumake *et al.*, 2001) and reduced hippocampal glucocorticoid receptor mRNA (Kabbaj *et al.*, 2000; Lachman *et al.*, 1993). However, I did not observe any differences in the PVH or corticosterone levels between helpless and non-helpless Holtzman rats that could account for the discrepancy in novelty-specific behavior. Cloninger (1987) argues that differences in the novelty-seeking trait reflect variation in the brains “incentive” or behavioral activation system that is mediated by the dopaminergic cell bodies originating from the ventral tegmental area and substantia nigra, and project to the neocortex, striatum and limbic structures such as the septum, nucleus accumbens and amygdala. Therefore, alterations in the nigrostriatal and mesolimbic dopamine systems may be more closely linked to novelty-specific activity among Holtzman rats.

However, changes in monoamine transmission may lead to an impaired stress-endocrine response such as the one observed in the congenitally helpless rats, especially since dopamine is known to play a neuromodulatory role in the stress response (Horger & Roth, 1996; Moghaddam, 2002). In general, it could be summarized that novelty-seeking behavior appears to be an important determinant in conferring vulnerability to psychopathologies as evidenced by findings in human and animal studies (Cloninger, 1987; Colorado *et al.*, 2006; Padilla *et al.*, 2009; Richman & Frueh, 1997; Shumake *et al.*, 2005; Stead *et al.*, 2006; Vollmayr *et al.*, 2004; Wang *et al.*, 1997). In addition, the combination of novelty-specific activity and heart rate accounted for over 35% of the variance in the escape response. It appears that novelty-induced activity and vulnerability to helplessness may be associated with an altered autonomic tone. This finding supports growing evidence that cardiovascular reactivity is related to an individual's unique behavioral response to the environment (Jemerin & Boyce, 1990).

Discovery of the diverse neurophysiologic variables that are related to stress vulnerability supports the complexity of psychiatric disorders. This complexity may be part of the reason why, in general, diagnoses are based on the presence of certain symptoms for a determined amount of time. Small open and controlled studies have shown that systematic treatment approaches can increase remission rates and improve treatment outcomes (Adli *et al.*, 2006).Transitioning into this type of practice could also have great economic impact. For example, treatment-resistant patients have incurred in six times the total medical costs compared to non-resistant patients ($42,344 versus $6,512) (Crown *et al.*, 2002). Standardized evidence-based guidelines that incorporate objective criteria such as brain, behavior, genetics and cardiovascular measures could enhance patient outcomes, reduce apparent treatment resistance, increase the quality of care, and potentially decrease direct and indirect health care costs.

6.10 CONCLUSION

This body of research has provided several unique insights. One is that commercially-available animal models have certain behavioral predispositions that can favor an investigator's interest. However, ignorance of these phenotypes can bias or negatively affect results. Another insight is that the Holtzman rat strain is potentially the first animal model of treatment resistant depression. Further detailed behavioral and brain mapping data is needed to validate this model of SSRI non-responsiveness, but eventually, one might use this model to identify behavioral or biological markers which predict which animals will be fluoxetine responders or non-responders and additional tests can be performed to see if the phenotype of responsiveness carries over to other classes of medications. Furthermore, for those interested in investigating the molecular mechanisms which confer this antidepressant resistance, selective breeding can be used to create a line of fluoxetine responders and a line of fluoxetine non-responders. Finally, bio-behavioral factors such as novelty-specific activity, heart rate measures, regional metabolism and functional connectivity between key prefrontal cortical and subcortical structures, such as the lateral habenula, carry high association with stress vulnerability. Analysis of these objective criteria in clinical practice could potentially have tremendous impact on patient treatment outcome and the health care economy.

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Vita

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